PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : A61K 31/33, 31/555, C07F 5/00, 5/06, 7/10, 15/02 — A1

(11) International Publication Number: WO 96/40108

(43) International Publication Date: 19 December 1996 (19.12.96)

(21) International Application Number: PCT/US96/10079

(22) International Filing Date: 7 June 1996 (07.06.96)

(30) Priority Data:
08/487,991 7 June 1995 (07.06.95) US

(60) Parent Application or Grant
(63) Related by Continuation
US 08/487,991 (CIP)
Filed on 7 June 1995 (07.06.95)

(71) Applicant *(for all designated States except US)*: WASHINGTON UNIVERSITY [US/US]; One Brookings Drive, St. Louis, MO 63130 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: PIWNICA-WORMS, David, R. [US/US]; 45 Godwin Lane, St. Louis, MO 63124 (US). SHARMA, Vijay [CA/US]; 9940 Warshire Drive, St. Louis, MO 63132 (US).

(74) Agents: FOX, Samuel, L. et al.; Sterne, Kessler, Goldstein & Fox, P.L.L.C., Suite 600, 1100 New York Avenue, N.W., Washington, DC 20005-3934 (US).

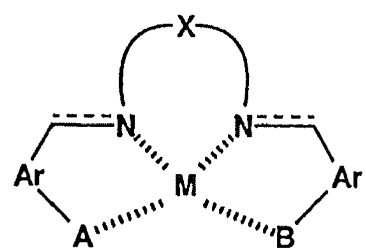
(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: MULTIDENTATE METAL COMPLEXES AND METHODS OF MAKING AND USING THEREOF

(57) Abstract

The invention relates to multidentate metal complexes having formula (I) wherein M is Fe, In, Ga or Al; the dashed lines represent independently a sigle or a double bond; the hatched lines represent coordination to the metal cation (M); A and B are oxygen, or in the case where Ar is 2-pyridyl or 2-pyrrolyl, a shared pair of electrons on the nitrogen; Ar is optionally substituted phenyl, optionally substituted naphthyl, optionally substituted 2-pyridyl or optionally substituted 2-pyrrolyl, with the proviso that at least one of the substituents comprises a boron atom; X is alternatively (i) $(CHR^2)_p[NR^3(CHR^4)_q]_r$, wherein p and q are independently 1, 2, 3, 4, 5 or 6; r is 0, 1 or 2; and R^2, R^3 and R^4 are independently hydrogen, lower alkyl or phenyl, or two adjacent R^2 or R^4 groups represent a double bond or a fused benzene ring where p or q, respectively, is greater than 2; with the proviso that where r is 1 or 2, then there are 1 or two additional sites of coordination to the metal; or (ii) optionally substituted phenyl, wherein the phenyl, when substituted, has lower alkyl substituents, optionally with: proviso (A), wherein at least one of the substituents of Ar comprises boron, or with proviso (B), wherein at least one of substituents of Ar comprises a linkage to a pharmaceutically active substituent.



(I)

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	GB	United Kingdom	MW	Malawi
AT	Austria	GE	Georgia	MX	Mexico
AU	Australia	GN	Guinea	NE	Niger
BB	Barbados	GR	Greece	NL	Netherlands
BE	Belgium	HU	Hungary	NO	Norway
BF	Burkina Faso	IE	Ireland	NZ	New Zealand
BG	Bulgaria	IT	Italy	PL	Poland
BJ	Benin	JP	Japan	PT	Portugal
BR	Brazil	KE	Kenya	RO	Romania
BY	Belarus	KG	Kyrgystan	RU	Russian Federation
CA	Canada	KP	Democratic People's Republic of Korea	SD	Sudan
CF	Central African Republic			SE	Sweden
CG	Congo	KR	Republic of Korea	SG	Singapore
CH	Switzerland	KZ	Kazakhstan	SI	Slovenia
CI	Côte d'Ivoire	LI	Liechtenstein	SK	Slovakia
CM	Cameroon	LK	Sri Lanka	SN	Senegal
CN	China	LR	Liberia	SZ	Swaziland
CS	Czechoslovakia	LT	Lithuania	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	LV	Latvia	TJ	Tajikistan
DK	Denmark	MC	Monaco	TT	Trinidad and Tobago
EE	Estonia	MD	Republic of Moldova	UA	Ukraine
ES	Spain	MG	Madagascar	UG	Uganda
FI	Finland	ML	Mali	US	United States of America
FR	France	MN	Mongolia	UZ	Uzbekistan
GA	Gabon	MR	Mauritania	VN	Viet Nam

Multidentate Metal Complexes and Methods of Making and Using Thereof

Field of the Invention

The invention relates to multidentate metal complexes, and their use in therapeutic treatments.

Background of the Invention

Metal complexes of multidentate ligands, in general (Kennard, *Inorg. Chim. Acta 2*:347 (1967); Liu *et al.*, *Inorg. Chem. 31*:5400 (1992)), and multidentate Schiff base ligands, in particular (Holm *et al.*, *Prog. Inorg. Chem. 1*:83 (1966); Patterson and Holm, *Bioinorg. Chem. 4*:257 (1975); Jurisson *et al.*, *Inorg. Chem. 23*:4743 (1984); Green *et al.*, *J. Am. Chem. Soc. 106*:3689 (1984)), have been known and studied for many years. The magnetic, electronic, and electrochemical properties of these metal complexes have been characterized and found to depend significantly on the nature of the substitutions on the backbone

of the Schiff base ligand (Holm *et al.*, *Prog. Inorg. Chem. 1*:83 (1966)). Metal complexes of Schiff base ligands containing linear N_4O_2 donor groups (Das Sarma and Ballar, *J. Am. Chem. Soc. 76*:4051 (1954); Das Sarma *et al.*, *J. Am. Chem. Soc. 86*:14 (1964); Tweedle and Wilson, *J. Am. Chem. Soc. 98*:4824 (1976); Sinn *et al.*, *J. Am. Chem. Soc. 100*:3375 (1978); Evans and Jakubovic, *Polyhedron 7*:1881 (1988); Chandra and Chakravorty, *Inorg. Chem. 31*:760 (1992); Tsang *et al.*, *J. Nuc. Med. 34*:1127 (1993)) and of analogous amine phenol ligands with linear N_4O_2 groups (Wong *et al.*, *Inorg. Chem. 34*:93 (1995)) have also been reported. Prior biomedical interest in all of these multidentate ligands and corresponding metal complexes has been generated by their potential use as radiopharmaceuticals and as chelating agents for use in treatment of metal overload states and toxicity.

Malaria is one of the most serious and widespread infectious diseases, resulting annually in more than 400 million cases and 2.5 million deaths worldwide, primarily in tropical countries (Muller and Baker, Medical Parasitology, Gower Medical Publishing, London (1990)). In addition, increased import of malaria into the United States in recent years by travelers, immigrants, and military personnel has generated a resurgence of national interest in this disease. Of the several malaria species infecting humans, the most abundant and fatal is *Plasmodium falciparum*, the causative organism in tertian malaria. Over the last several decades much research and development has been invested in a wide range of antimalarial agents.

Antimalarial chemotherapy has historically targeted prophylactic, suppressive treatment and/or clinical cure. Antimalarial agents can be roughly divided into several distinct groups based on mechanisms of action. The first group, characterized by older, well known agents such as chloroquine, primaquine, quinine and their derivatives, demonstrates rapid cytological action. Of this group, chloroquine is the most common, well-tolerated, and cost-effective drug for prophylaxis and therapy of malaria (Muller and Baker, *Medical Parasitology*, Gower Medical Publishing, London (1990)).

These agents are thought to selectively accumulate within the parasite and interfere with digestive vacuole function. In fact, chloroquine-sensitive strains of *Plasmodium* parasites concentrate chloroquine approximately 800-fold in their digestive vacuoles (Veignie and Moreau, *Ann. Trop. Med. Parasitol. 85*:229 (1991)). The digestive vacuole is a crucial organelle for the parasite, responsible for degradation of the host's hemoglobin, the main source of nutrients for the parasite. The digestive vacuole has lysosomal characteristics such as an acid pH and a high content of acid hydrolases (Goldberg and Slater, *Parasitol. Today 8*:280 (1992)). Hemoglobin degradation inside the digestive vacuole generates large amounts of toxic heme which is detoxified by polymerization into pigment bodies by heme polymerase, an enzyme found exclusively in the digestive vacuole. Chloroquine inhibits the heme polymerase *in vitro* at concentrations which are found in the digestive vacuole of chloroquine-sensitive parasites, explaining the observation that accumulation of chloroquine in the digestive vacuole disturbs the formation of pigment bodies (Slater and Cerami, *Nature 355*:167 (1992) (London)). However, chloroquine-resistant organisms are now reported from every major region in which malaria is endemic (van Es *et al.*, *Clin. Invest. Med. 16*:285 (1993)).

The second major group of agents, represented by chloroguanide, pyrimethamine, sulfonamides, and derivatives, has mechanisms of action that are slower in onset and target the synthesis of folinic acid (folate) from para-aminobenzoic acid (PABA). These agents tend to interfere with the incorporation of PABA into folate, a synthetic process which does not occur in host mammalian cells, or to inhibit parasitic dihydrofolate reductase. Acquired or natural drug resistance to these agents can be demonstrated readily in the laboratory and, like the first group, has been reported from every major endemic malarial region.

Several other approaches to anti-malarial therapy are known. U.S. Patent No. 5,270,037 discloses the treatment of malaria with compositions comprising an interferon.

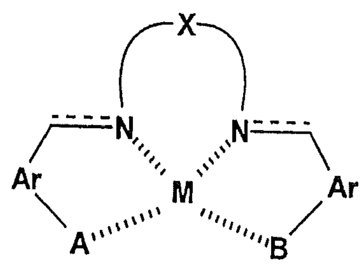
In addition, there are a number of publications which teach the treatment of malaria with certain free ligands of iron chelators. See, for example, WO93/00082 discloses the use of hydroamate derivatives which are useful for removal of iron (III) from mammalian cells and treatment of disorders caused by pathogenic organisms, such as *Plasmodium falciparum* that causes malaria. *See also*, EPA 214,933 EPA 214,101, Iheanacho *et al., Trans. R. Soc. Trop. Med. Hyg. 84*:213-216 (1990), Stahel *et al., Am. J. Trop. Med. Hyg. 39*:236-240 (1988), Hershko and Peto, *J. Med. Chem. 168*:375-387 (1988), and Hershko *et al., J. Inorg. Biochem. 47*:267-277 (1992), which teach the use of other iron (III) chelators for the treatment of malaria.

Thus, there exists urgent need to discover and develop novel anti-malarial agents that are effective against *Plasmodium* species, especially emerging drug-resistant *P. falciparum* strains (Sharma, *Nature 373*:279 (1995)).

Summary of the Invention

The present invention arises from the inventors' discovery that certain intact metal complexes comprising multidentate ligands are effective as anti-malarial agents. Thus, the invention relates to new compositions of matter that can be used for treating malaria and treatment of malaria with these compositions. In addition, the complexes can be used in the treatment of other diseases as described herein, such as cancer and diseases attributed to multidrug resistance transporters. Thus, the invention also relates to pharmaceutical compositions containing the complexes of the invention.

In particular, the invention also relates to a multidentate cationic metal complex having the following formula ***I***:



I

wherein

M is Fe, In, Ga or Al;

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

A and B are oxygen, or in the case where Ar is 2-pyridyl or optionally substituted 2-pyrrolyl, a shared pair of electrons on the nitrogen;

Ar is optionally substituted phenyl, optionally substituted naphthyl, optionally substituted 2-pyridyl or optionally substituted 2-pyrrolyl; and

X is $(CHR^2)_p[NR^3(CHR^4)_q]_r$, wherein p and q are independently 1, 2, 3, 4, 5 or 6; r is 0, 1 or 2; and R^2, R^3 and R^4 are independently hydrogen, lower alkyl or phenyl, or two adjacent R^2 or R^4 groups represent a double bond or a fused benzene ring where p or q, respectively, is greater than 2;

with the proviso that where r is 1 or 2, then there are 1 or two additional sites of coordination to the metal.

The complex optionally further comprises $^-A^-$, where ^-A is a pharmaceutically acceptable anion.

The invention also relates to a multidentate cationic metal complex according to formula ***I*** with proviso (A), wherein at least one of the substituents of Ar comprises boron.

The invention also provides a multidentate cationic metal complex according to formula *I* with proviso (B), wherein at least one of substituents of Ar comprises a linkage to a pharmaceutically active substituent.

The invention also provides a a multidentate cationic metal complex according to formula *I*, wherein X is optionally substituted phenyl. X, when having a substituted phenyl, has substituents as lower alkyl groups, with methyl groups preferred at the meta and para positions relative to the N bonds.

The invention further relates to a multidentate cationic metal complex selected from the group consisting of N,N′-bis[3-(2-hydroxy-3-methoxybenzylimino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-ethoxybenzylimino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-methoxybenzylimino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-methoxy-5-bromobenzylimino)propyl]-ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-5-chlorobenzylimino)-propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-methoxybenzyl-amino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-ethoxybenzylamino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-4,6-dimethoxybenzylamino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-methoxy-5-bromobenzylamino)propyl]ethylene-diamine $M(III)^+A^-$, and N,N′-bis[3-(2-hydroxy-5-chlorobenzylamino)-propyl]ethylenediamine $M(III)^+A^-$, wherein M(III) is Fe, In, Ga or Al, and A^- is a pharmaceutically acceptable anion.

The invention also relates to a multidentate cationic metal complex selected from the group consisting of N,N′-bis[3-(2-hydroxy-3-fluoro-benzylimino)propyl]ethylene-diamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-3-fluorobenzylamino)propyl]-ethylene diamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-5-bromobenzyl-imino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-5-bromobenzylimino)propyl]ethylenediamine $M(III)^+A^-$, N,N′-bis[3-(2-hydroxy-5-bromobenzylamino)propyl]ethylenediamine $M(III)^+A^-$, wherein M(III) is Fe(III), In(III), Ga(III), or Al(III), and A^- is a pharmaceutically acceptable anion.

The invention also relates to a pharmaceutical composition comprising any of the complexes of the invention, and a pharmaceutically acceptable carrier.

The invention also relates to a method for treating malaria comprising administering an effective amount of a multidentate cationic metal complex according to formula *I*.

The invention also relates to a method of treating cancer, comprising administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to formula *I*.

The invention also relates to a method for the treatment of diseases attributed to the multidrug family of transporters, comprising administrating to an animal in need thereof an effective amount of a multidentate cationic metal complex according to formula *I*, wherein formula *I* with proviso (A) is preferred, wherein at least one of the substituents of Ar comprises boron.

The invention also relates to a method of potentiating photodynamic therapy in the treatment of cancer, comprising (a) administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to formula *I*, and (b) exposing cancer cells of the animal to wavelengths of light effective to kill said cancer cell.

The invention also relates to a method of treating cancer by boron-neutron therapy, comprising (a) administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to formula *I*; and (b) exposing cancer cells of said animal to a neutron beam effective to kill said cancer cells.

The invention also relates to a method of enhancing the oral absorption or cell accumulation of a pharmacologically active substance, comprising administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to formula *I*, with proviso (B), wherein at least one of substituents of Ar comprises a linkage to a pharmaceutically active substituent.

In preferred embodiments of all of the complexes described herein, the hatched lines represent coordination to the metal (III) cation (M).

Brief Description of the Figures

Figure 1: Concentration-effect curves of substituted MENPBI-Fe(III) complexes on *P. falciparum* growth inhibition. ■ = 3-OMe MENPBI-Fe(III); □ = 3-OEt MENPBI-Fe(III); and ● = 4,6-di-OMe MENPBI-Fe(III).

Figure 2: Cytotoxicity of a MENPBI complex in KB-3-1 and KB-8-5 Cells. Figure 2 depicts a graph showing the concentration effects of a substituted MENPBI-Fe(III) complex on cytotoxic activity in human renal cell carcinoma drug-sensitive cells (KB-3-1) and derived multidrug-resistant cells (KB-8-5), wherein ○, ▿ = parental KB-3-1 cells; ●, ▾ = multidrug resistant KB-8-5 cells; ○, ● = MEMPBI-Fe(III) complex; and ▿, ▾ = colchicine cytotoxic control.

Figure 3: Structure of the hexadentate (N_4O_2) ethylenedi-amine-bis(propyl(R-benzylimino)) ligand metal(III) monocationic complex shown in the trans phenolic configuration.

Figure 4A-B: Effect of 4,6-dimethoxy-ENBPI Fe(III) complex on intraerythrocytic *P. falciparum* in culture. (***Figure 4A***): Concentration-effect curve of antimalarial activity: chloroquin sensitive (HB3) and resistant (FCR-3, Indo-1, Dd2) strains were grown in the absence or presence of various concentrations of inhibitor. Growth inhibition was measured by the ^{3}H-hypoxanthine incorporation assay. Data are shown as mean values of triplicate determinations; error bars (when larger than symbol) represent ± SEM. (***Figure 4B***): Time course of antimalarial activity: parasites at the early (ring) stage (a) were incubated with 5 μM inhibitor or with the DMSO vehicle. By 14 hrs (b), treated parasites had started to fill in their cytoplasm and had matured to the early trophozoite stage. At 24 hrs (c), treated parasites had not matured further and abnormal forms were seen. No hemozoin formation was apparent. By 30 hrs (d), the few remaining parasites were pyknotic. In contrast, control parasites had

matured to the late trophozoite stage by 24 hrs (e), and large digestive vacuole filled with hemozoin was observed. By 30 hrs (f), control parasites had undergone normal schizogony. Again, a hemozoin-replete vacuole was seen.

Figure 5: Inhibition of heme polymerization by 4,6-dimethoxy-ENBPI Fe(III) complex. Heme polymerization was assayed by pre-formed hemozoin nucleation (open circles) or histidine-rich protein II-facilitated reaction (closed circles) in the presence of various concentrations of drug. Data are from representative experiments; values are the means of two independent determinations.

Figure 6: General structure of hexadentate R-ENBPI ligands.

Figure 7: Pathway for the synthesis of R-ENBPI metal complexes.

Figure 8A-B: Proton decoupled ^{13}C NMR spectra (75.4 MHz) of H_3Mabi (1) (***Figure 8A***); and H_3DMabi (2) (***Figure 8B***) in $CDCl_3$ at room temperature. Chemical shifts are reported in ppm referenced to TMS.

Figure 9A-L: Cell survival studies and LC_{50} determination. Survival of parental KB-3-1 (O, (triangle), □) and multidrug resistant KB-8-5 (●, ▼, ■) cells in increasing concentrations of R-ENBPI metal(III) complexes (O, ●), 25 μM colchicine ((triangle), ▼) or metal(III) ions (□, ■). ***Figure 9A-D, 9E-H, 9I-L*** represent Al(III), Fe(III), Ga(III), and In(III) complexes of: (i) 3-MeO-R-ENBPI (***Figure 9A-D***), (ii) 4,6-diMeO-R-ENBPI (***Figure 9E-H***); and (iii) the free metals (***Figure 9I-L***), respectively. Each point represents the mean of triplicate determinations; bars represent ± SEM when larger than the symbol.

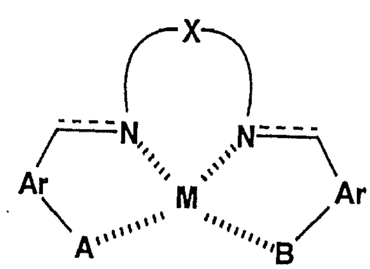
Detailed Description of the Preferred Embodiments

The invention is related to the discovery that intact metal complexes comprising multidentate ligands are useful for treating or preventing malaria. The multidentate ligands that may be used in the present invention include the Schiff base imine that is obtained by reacting an optionally substituted aryl, 2-pyridyl, or pyrrolyl aldehyde with an amino compound comprising at least two

primary or secondary amines, as well as the reduced amine obtained by reducing the imines with borohydride. Preferably, the multidentate ligands are bi- or polydentate salicylaldiamines.

A wide variety of multidentate ligands have been prepared in the art. For example, see Kennard, *Inorg. Chim. Acta 2*:347 (1967); Liu *et al.*, *Inorg. Chem. 31*:5400 (1992), Holm *et al.*, *Prog. Inorg. Chem. 1*:83 (1966); Patterson and Holm, Bioinorg. Chem. 4:257 (1975); Jurisson *et al.*, *Inorg. Chem. 23*:4743 (1984); Green *et al.*, *J. Am. Chem. Soc. 106*:3689 (1984); Holm *et al.*, *Prog. Inorg. Chem. 1*:83 (1966); Das Sarma and Ballar, *J. Am. Chem. Soc. 76*:4051 (1954); Das Sarma *et al.*, *J. Am. Chem. Soc. 86*:14 (1964); Tweedle and Wilson, *J. Am. Chem. Soc. 98*:4824 (1976); Sinn *et al.*, *J. Am. Chem. Soc. 100*:3375 (1978); Evans and Jakubovic, *Polyhedron 7*:1881 (1988); Chandra and Chakravorty, *Inorg. Chem. 31*:760 (1992); Tsang *et al.*, *J. Nuc. Med. 34*:1127 (1993), and Wong *et al.*, *Inorg. Chem. 34*:93 (1995).

The disclosed multidentate ligand metal complexes can have the formula ***I***:



I

wherein

M is Fe, In, Ga or Al;

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

A and B are oxygen or nitrogen, or in the case where Ar is 2-pyridyl or 2-pyrrolyl, a shared pair of electrons on the nitrogen;

Ar is optionally substituted phenyl, naphthyl, 2-pyridyl or 2-pyrrolyl;

X is $(CHR^2)_p[NR^3(CHR^4)_q]_r$, wherein R^2, R^3 and R^4 are independently hydrogen, lower alkyl or phenyl, or wherein two adjacent R^2 or R^4 groups represent a double bond or a fused benzene ring where p or q, respectively, is greater than 2; p and q are independently 1, 2, 3, 4, 5 or 6; and r is 0, 1 or 2; with the proviso that where r is 1 or 2, then the multidentate ligand coordinates 5 or 6 times to the metal.

The complex optionally further comprises $-A^-$, where ^-A is a pharmaceutically acceptable anion.

In Formula *I*, X can alternatively be optionally substituted phenyl, wherein the phenyl, when substituted, has lower alkyl substituents, with methyl groups preferred at the meta and para positions relative to the N bonds.

Optional substituents of Ar optionally further comprise a boron atom (as proviso (A)) or a linkage to a pharmaceutically active substance (as proviso (B)).

Preferably, the multidentate ligands fall within the general category of hexadentate N_4O_2 amine phenol ligands and the analogous Schiff base phenol ligands. Such complexes may be referred to as M[ethylene diamine N,N′-bis(propyl)(2-hydroxy R-benzylamino)] complexes or "MENPBA-complexes" and M[ethylene diamine N,N′-bis(propyl)(2-hydroxy R-benzylimino)] complexes or "MENPBI-complexes", where R represents alkyl or substituted alkyl or aryl or substituted aryl moieties and M represents any metal conferring the desired hexadentate coordination chemistry and biological activities. The components of the alkylene backbone scaffold could readily contain unsaturated carbon chains and still confer the desired properties. The most preferred embodiments contain R = OMe and/or halogen moieties and M = Fe(III). The preferred complexes are overall (1+) monocations. For an example of a representative general procedure for the preparation of MENPBI-complexes, the synthesis of MENPBI-Fe(III) is described below.

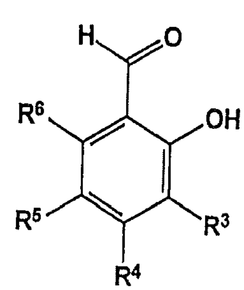
The anion counter ion of the metal complexes of the present invention can alternatively be any pharmaceutically acceptable anion including chloride,

bromide, iodide, sulfate, phosphate, acetate, fumarate, succinate, citrate, tartrate, and the like.

The multidentate ligand complexes may be prepared as follows. In general, the method involves the condensation of a salicylaldehyde, a 2-pyridinecarboxaldehyde, or a 2-pyrrolidinecarboxaldehyde, with a diamine to give a Schiff base. The Schiff base itself can be complexed with a metal salt and administered as an anti-malarial, anti-cancer agent or pharmaceutical enhancer, or reduced with sodium borohydride or cyanoborohydride to give the resulting amine, which may also be complexed with the metal salt and used as an antimalarial, anti-cancer agent or pharmaceutical enhancer.

Non-limiting examples of diamines which can be used in the present invention include, but are not limited to ethylenediamine, bis(3-aminopropyl)ethylenediamine, bis(3-aminopropyl)-1,3-propanediamine, 1,3-bis(aminomethyl)cyclohexane, bis(2-aminoethyl)-1,3-propanediamine, bis(2-aminopropyl)-1,4-butanediamine, bis(2-aminopropyl)ethylenediamine, bis(2-aminoethyl)ethylenediamine, 1,5,9-triazanonane, 1,4,9,12-tetraazododec-6-ene, 1,2-propylenediamine, 1,5-pentanediamine, 1,6-hexanediamine, 2-methyl-2-aminoethylamine, 3-methyl-3-aminopropylamine, 4-methyl-4-aminobutylamine, 5-methylaminopentylamine, 6-methylaminohexylamine, 3-methylamino-2-methylpropylamine, 2-ethylaminoethylamine, 3-ethylaminopropylamine, 4-ethylaminobutylamine, 5-ethylaminopentylamine, 6-ethylaminohexylamine, 3-propylaminopropylamine,4-propylaminobutylamine,5-propylaminopentylamine, and 6-propylaminohexylamine.

Examples of salicylaldehyde derivatives which may be used have the formula ***II***:

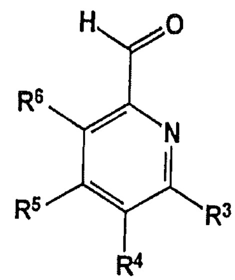


II

wherein R^3, R^4, R^5 and R^6 are independently hydrogen, halo, alkyl, alkoxy or nitro.

Specific examples include 5-bromosalicylaldehyde, 5-chlorosalicylaldehyde, 3-fluorosalicylaldehyde, 3-methoxysalicylaldehyde, 3-ethoxysalicylaldehyde, 4,6-dimethoxysalicylaldehyde, 5-bromo-3-methoxysalicylaldehyde and 5-nitrosalicyladehyde, which are commercially available, as well as salicylaldehyde-5-sulfonate described by Evans and Jakubovic, *Polyhedron* 7:1881-1889 (1988), 4,6-dimethoxysalicylaldehyde described by Tsang *et al., J. Nucl. Med. 34*:1127-1131 (1993), and x-nitrosalicyladehyde (apparently the position of the nitro group was not known) described by Tweedle and Wilson, *J. Amer. Chem. Soc. 98*:4824-4834 (1976). For a review on the preparation of salicylaldimine complexes see Holm *et al., Prog. Inorg. Chem. 1*:83 (1966).

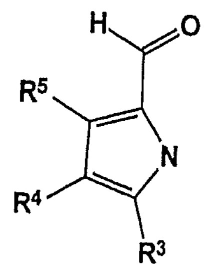
Examples or pyridocarboxaldehydes which may be used have the formula *III*:



III

wherein R^3, R^4, R^5 and R^6 are independently hydrogen, halo, alkyl, alkoxy or nitro.

Examples or pyrrolidinecarboxaldehydes which may be used have the formula *IV*:



IV

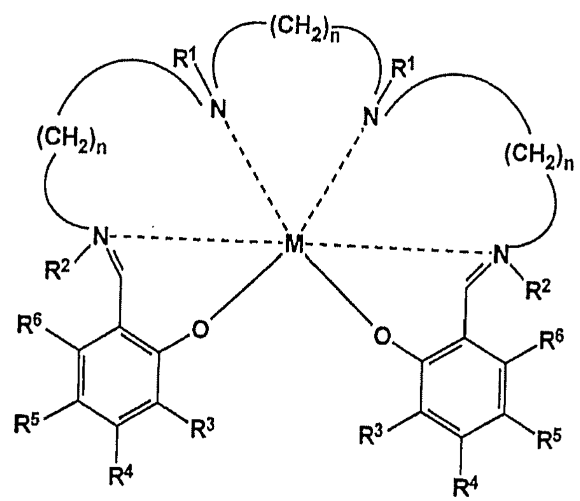
wherein R^3, R^4, R^5 and R^6 are independently hydrogen, halo, alkyl, alkoxy or nitro.

For example, bis(3-aminopropyl)ethylenediamine and the corresponding substituted salicylaldehyde, pyridocarboxaldehyde, or pyrrolidine-carboxaldehyde (1:2 ratio) are dissolved in methanol, stirred for 30 minutes, and treated with dropwise addition of sodium methoxide dissolved in methanol. After 30 minutes, the solution is treated with dropwise addition of M(III) salt (e.g., hydrated ferric nitrate) dissolved in methanol, stirred for 1 hour, and then filtered. Volatiles are removed under reduced pressure, residue is dissolved in hot water, and potassium hexafluorophosphate dissolved in water is added. Volatiles are removed under reduced pressure and the desired substituted MENPBI-M(III)

(e.g., MENPBI-Fe(III)) residue is crystallized from water and acetone. Analysis and characterization of substituted free ligands and M^+-complexes may be performed by routine analytical ^{1}H-NMR, ^{13} C-NMR, and/or GC-mass spectrometry.

The following preferred EMPBI or ENPBA complexes according to formulae *V-XII* can have overall neutral or positive charge, and when charged and optionally include A^-, as a pharmaceutically acceptable anion.

Preferred monocationic substituted ENPBI-complexes according to the present invention have the following formula *V*:



V

wherein:

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

R^1 and R^2 are independently hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group; and

n = 2, 3, 4, 5 or 6.

In especially preferred compounds, R^3 is methoxy or R^3 is ethoxy, or R^4 and R^6 are methoxy.

By the term "lower alkoxy" is intended C_{1-6} alkoxy groups.

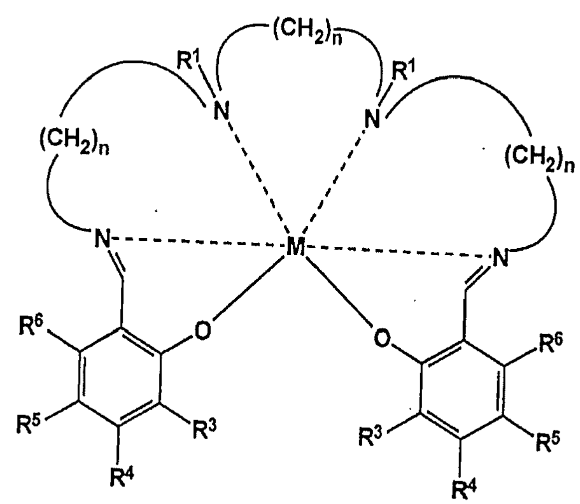
By the term "halo" is intended fluoro, chloro, bromo or iodo.

By the term "alkyl" is intended a straight or branched chain hydrocarbon group, preferably containing one to six carbon atoms.

Preferred R^3 groups include OCH_3, OC_2H_5, OC^3H^7, and halogens.

Preferred R^4 and R^6 groups are OCH_3 and OC_2H_5.

Preferred substituted metallo[alkylene diamine N,N'-bis(alkyl)(2-hydroxy R-benzylimino)] complexes have formula *VI*:



VI

wherein:

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent independently a single or a double bond;

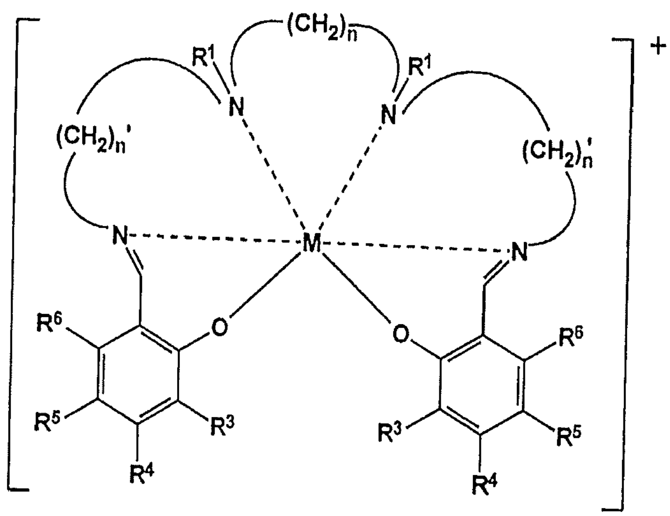
the hatched lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group; and

n = 2, 3, 4, 5 or 6.

Especially preferred substituted MENPBI-complexes have the following formula *VII*:



VII

wherein:

M is Fe or Al;

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

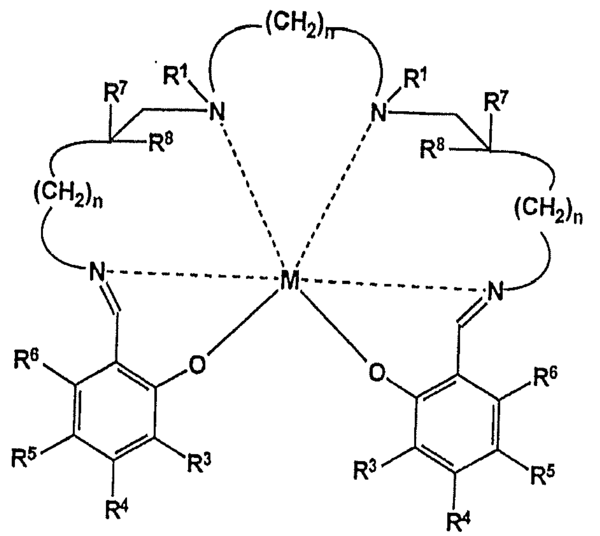
R^3 is OCH_3 or OC_2H_5;

R^4 and R^6 are OCH_3;

R^1 and R^5 are H; and

n is 2; n′ is 3;

Other preferred ENPBI complexes have the following formula *VIII*:



VIII

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3} or Al^{+3};

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

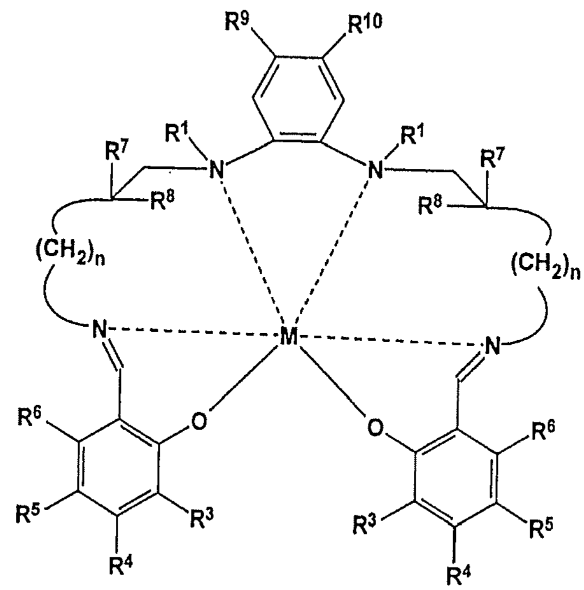
R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7 and R^8 are independently hydrogen or lower alkyl; and

n = 2, 3, 4, 5 or 6.

Other preferred ENPBI complexes have the following formula *IX*:



IX

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

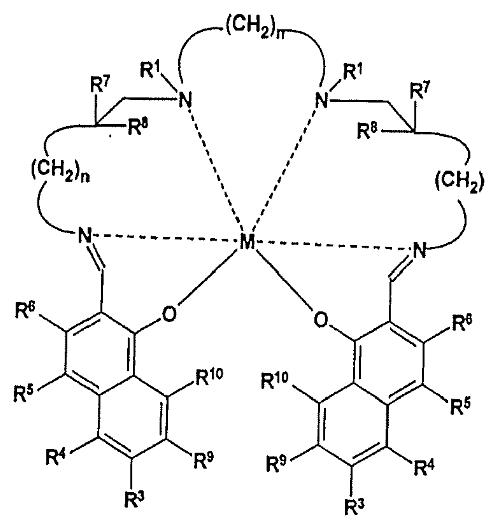
R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7, R^8, R^9 and R^{10} are independently hydrogen or lower alkyl; and

n = 1, 2, 3, 4, 5 or 6.

Other preferred ENPBI complexes have the following formula *X*:



X

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

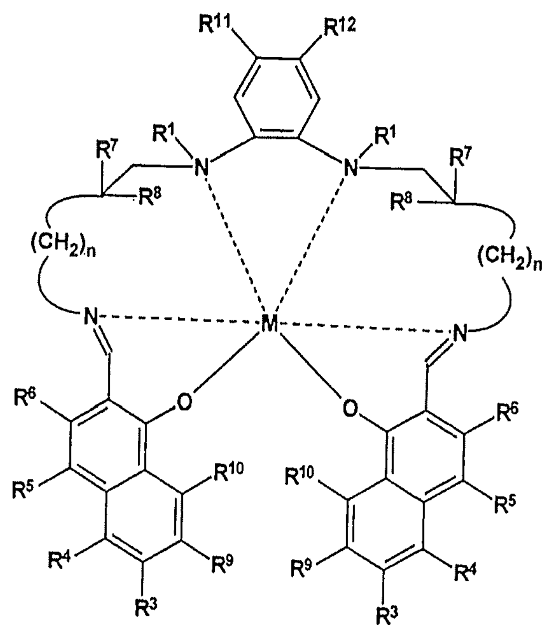
R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7 and R^8 are independently hydrogen or alkyl;

R^9 and R^{10} are independently hydrogen, a lower alkyl, a lower alkoxy, halo or nitro group; and

n = 1, 2, 3, 4, 5 or 6.

Other preferred ENPBI complexes have the following formula *XI*:



XI

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

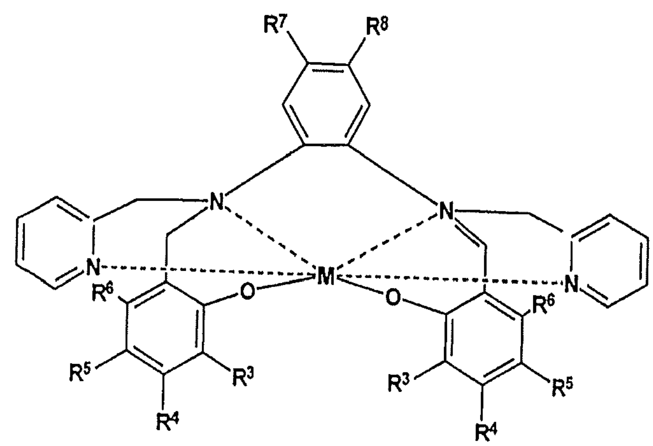
R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7, R^8, R^{11} and R^{12} are independently hydrogen or lower alkyl;

R^9 and R^{10} are independently hydrogen, a lower alkyl, a lower alkoxy, halo or nitro group; and

n = 1, 2, 3, 4, 5 or 6; and wherein R^{11} and R^{12} are preferably methyl.

Other preferred ENPBI complexes have the following formula *XII*:



XII

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7 and R^8 are independently hydrogen or lower alkyl; and

n = 2, 3, 4, 5 or 6.

Especially preferred substituted MENPBA-complexes have structures analogous to formulas *I, V, VI, VIII, IX, X, XI*, and *XII*, but the Schiff base is reduced to an amine.

The metal complexes of the present invention may be co-administered with one or more known antimalarial substances. The known antimalarial substances can be divided into the following 6 main groups on the basis of their chemical compositions:

1) the 9-aminoacridines (e.g., mepacrine),
2) the 4-aminoquinolines (e.g., amodiaquine, chloroquine, hydroxychloroquine),
3) the 8-aminoquinolines (e.g., primaquine, quinocide),
4) the biguanides with an inhibiting effect on dihydrofolic acid reductase (e.g., chloroproguanil, cycloguanil, proguanil),
5) the diaminopyrimidines (e.g., pyrimethamine),
6) the quinine salts.

In addition to these groups, sulphones such as dapsone, sulphonamides, sulphanilamides and antibiotics such as tetracycline may also be used as antimalarial agents.

Depending on their mode of activity the known antimalarial agents can be divided into the following categories:

1. causal prophylactic substances effective against primary tissue stages,
2. active substances directed against relapses or recurrences and effective against latent tissue stages,
3. blood schizonticides,
4. gametocytocides and
5. sporonticides.

The first group includes, for example, proguanil, pyrimethamine and primaquine and derivatives thereof, and also sulphanilamides, sulphonamides and tetracyclines. The second group includes, for example, 8-aminoquinolines, such as primaquine and its analogues and derivatives, floxacrine, cycloguanil, dapsone and quinazolines. Substances active against the blood schizonts include, in particular, 4-aminoacridines such as mepacrine, and the 4-aminoquinolines such

as chloroquine or chloroquinesulphate, quinine, amodiaquine and mepacrine, mefloquine, and related compounds such as halofantrene, pyrimethamine, proguanil, primaquine and the sulphanilamides and sulphonamides, particularly in conjunction with pyrimethamine.

Other substances which may be coadministered are the sesquiterpene lactones based on the compound artemisinine, and the semisynthetic derivatives thereof, such as artesunate, artemether, piperaquine, hydroxypiperaquine, pyronaridine, halofantrene and, generally, the biguanides and quinine salts.

The schizonticides mentioned above are effective against the schizonts of, for example, *P. vivax, P. malariae* or *P. ovale*, but not against the mature gametocytes. The 8-aminoquinolines, such as primaquine and quinocide, are also effective against the gametocytes. Proguanil, primaquine and pyrimethamine are also sporonticidal agents. Other known antimalarial agents are: chloroproguanil, cycloguanil (e.g., as a salt of embonic acid), pamaquine, plasmocide, totaquine, spirogermanium, febrifugine, brusatol, bruceine-A, bruceine-B, bruceine-C, yadanziolide-A, tebuquine, enpirolin, eurycomanone, 3-(4-imidazolyl)-2-(pivaloylamido)-propionylhydrazide, cinchonidine; cucurbitacine, tripynadine, 5-ethylthioribose, arteether (ethylether analogues of artemether), artenilic acid, pyrexol, atalaphillinine, diformyldapsone, bruceantine, nitroquine, octanoylprimaquine, pyrimethamine plus sulfadoxine, hivernine, dabequine, artelinic acid, mefloquinquinate, halfantrin-beta-glycerophosphate, nimbolide, sergeolide (quassinoid of *Picrolemma pseudocoeffea*), simalikalactone-D, fluoroquine, fluorenmethanol, isouramil, cycloleucine, acedapsone (diacetyldapsone), gentiopicrine, amquinate (amquinolate), endochine, pentaquine, isopentaquine, methylchloroquine, amopyroquine, quinine, hydroquinine (dihydroquinine), dimeplasmine, azacrine, diapromine, menoctone, cycloquine (haloquine), lapinone, aristoquine, cloguanamil, clociguanil, brindoxime, cinchonine, tripiperaquine, 3-hydroxy-2-(4-(4-phenyl)-cyclohexyl)-1,4-anthraquinone, aminodiaquine, 4-methyl-5-n-pentoxyprimaquine, 4-methyl-5-n-hexoxyprimaquine, 2-(4-(4-chlorophenyl)-cyclohexyl)-3-hydroxy-1,4-

naphthalenedione, gossypol derivatives, halofantrine (1,3-dichloro-α-(2-(dibutylamino)ethyl)-6-(trifluoromethyl)-9-phentantrene-methanol), cinchona alkaloids (e.g., in the combination quinine, quinidine, cinchonine), N,N'-bis(3-((phenylmethyl)amino)propyl)-1,8-octanediamine, N,N-bis(3-((phenyl-methyl)amino)-propyl)-1,7-diaminoheptane, selenium-analogues of 2-acetyl and 2-propionyl-pyridinethiosemicarbazones, tebuquine, 2,6-bis(1-piperidinylmethyl)-4-((7-(trifluoromethyl)-4-quinolinyl)amino)-phenol, primary phosphoric acid esters of 4'-chloro-5-(1,1-dimethylethyl)-3-(((1,1-dimethylethyl)amino)methyl)-(1,1'-biphenyl-2-ol, N4-(2,6-dimethoxy-4-methyl-5-(3-trifluoromethyl)-phenoxy-8-quinolinyl)-1,4-pentanediamine, N,N-diethyl-N'-(6-methoxy-4-methyl-8-quinolinyl)-1,6-hexanediamine, 5-(N-aryl-tropan-3-yl)- and 5-(piperidin-4-yl)-2,4-diamino-pyrimidine, 4'-amino-4-n-propylamino-2-methyl-diphenylsulphone, 5-ethylthioribose, riboflavin-analogues, 1-(3-(2,4-dichlorophenoxy)-1,6-dihydro-6,6-dimethyl-1,3,5-triazine-2,4-diamine as the monohydrobromide, 1,6-dihydro-6,6-dimethyl-1-(3-(2,4,5-trichlorophenoxy)-propoxy)-1,3,5-triazine-2,4-diamine as the monohydrochloride, trans-2-(4-(1,1-dimethylethyl)-cyclohexyl)-3-hydroxy-1,4-naphthalenedione, enpiroline, mirincamycin, tripynadine, 3-(4-imidazolyl)-2-(pivaloylamido)propionylhydrazide, 2-acetylpyridine-thiosemicarbazones and the pyrrolidine derivatives thereof.

The present invention also relates to the use of these complexes for their cytotoxic activity on other cell types. Such cell types include cancer cells such as CNS tumors, breast cancer, lung, head and neck, cancer lymphoma, leukemias, ovarian carcinoma, sarcomas, renal cell carcinoma, and prostate cancer.

Thus, the invention is also related to a method of treating cancer comprising contacting said cells with an effective amount of one of the multidentate cationic metal complexes of the present invention. Such contacting will typically be carried out by administration *in vivo* to an animal.

The present invention also relates to the use of these metal complexes as potentiators of photodynamic therapy. Light-absorbing chemicals such as the

metal complexes of the present invention are selectively retained by cancer cells. The cancer cells are killed when exposed to certain wavelengths of light [(400 to 800 nm)]. Typically, the metal complexes are administered to the animal and the complex-treated cells are exposed to laser beams of the appropriate wavelengths through an endoscope. Such an approach works best with cancer cells that are localized (and not in circulation, e.g., leukemia cells) and where the cancer cells are easily accessible. *See*, Harrison's Principles of Internal Medicine, 11th. Edition, Braunwald *et al.* (eds.), McGraw-Hill Book Co, New York, N.Y., pp.441 (1987). Examples of such cancer cells include CNS tumors, breast cancer, lung, head and neck, lymphoma, and melanoma.

The present invention also relates to the use of these metal complexes which comprise boron atoms in boron neutron therapy. Such metal complexes can be prepared by reaction of a compound having formula ***III***, wherein one of R^3, R^4 or R^5 is a nitro group, with a reducing agent such as H_2/Pd or Sn/HCl to give the corresponding aniline. This amine may be coupled with the active ester of trimethylamine-borane carboxylic acid (an α-boron analog of betaine, available from Sigma), in the presence of a dehydration agent, to give the boron-substituted complex.

Alternatively, a catechol (Aldrich) can be converted to salicylaldehyde using protection of one hydroxy group through methoxymethylether (MOM) and the other hydroxy group as acetate (acetic anhydride) exploiting the ortho directing ability of MOM to make salicylaldehyde, and then deprotecting the acetate group (NaOH, MeOH). Subsequently, react with trialkyl borane and deprotect MOM in the final step.

Another alternative can be used when a ligand wraps around a metal. Treatment with equimolar sodium tetraphenylborate can lead to incorporation of boron into the molecule (Nozaki *et al.*, *Inorg. Chem. 34*:2108 (1995)).

Examples of the active esters which may be used in the practice of the invention include the hemi-succinate esters of N-hydroxysuccinimide, sulfo-N-hydroxysuccinimide, hydroxybenzotriazole, and p-nitrophenol. Examples of

dehydration agents include dicyclohexylcarbodiimide (DCC), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (EDC), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDCI). The use of EDC to form conjugates is disclosed in U.S. Patent No. 4,526,714, and PCT publication no. WO91/01750, and Arnon, R *et al., Proc. Natl. Acad. Sci. (USA)* 77:6769-6772 (1980), the disclosures of which are fully incorporated by reference herein.

Once prepared, the boron-containing complexes can be administered to a patient suffering, for example, from one of the tumors mentioned above. The complexes will be taken up by the cells. When exposed to neutron therapy, the boron atoms will absorb the neutrons and release the energy thereof, thereby killing the cancer cells.

The metal complexes of the present invention may also be used as pharmacologically active substance delivery tools to enhance oral absorption and cell accumulation of linked prodrugs. Preferably, the pharmacologically active substances are linked through the benzene ring to a peptide, oligonucleotide, peptidomimetic, peptide nucleic acid analog or derivative thereof. The lipophilic cationic properties of the metal complexes of the present invention provide a thermodynamic driving force that pull the linked pharmacologically active substance across membrane bilayers of cells, in response to the negative inner membrane potential.

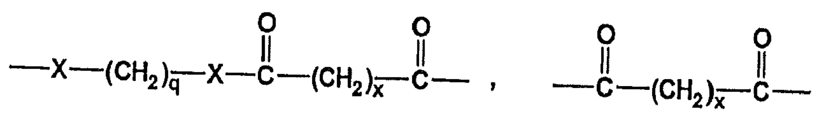
Thus, the invention also relates to the use of the complexes of the present invention in the preparation of pharmacologically active substance-complex conjugates which can be used to target cells. The conjugates may be prepared by reaction of the aniline mentioned above with a bifunctional linking group which is capable of linking the aniline amine to a pharmacologically active substance.

Thus, the present invention is also directed to complex/pharmacologically active substance conjugates which comprise a multidentate ligand-metal complex of the invention linked to a pharmacologically active substance, wherein the

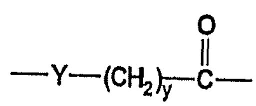
linkage does not interfere substantially with the ability of the pharmacologically active substance to carry out its expected pharmacological action.

By the term "linker group" is intended one or more bifunctional molecules which can be used to covalently couple the complex to the pharmacologically active substance and which do not interfere with the pharmacologic action of the pharmacologically active substance *in vivo*.

Examples of linker groups which can be used to link the complex to the pharmacologically active substance may comprise



wherein $q = 2\text{-}5$, $x = 2\text{-}12$; and



wherein Y is N or S, $y = 1\text{-}3$.

Alternatively, other salicylaldehyde derivatives can be synthesized by exploiting the presence of halogen at 5 position through coupling reaction involving protected derivative (Formula II) and 3-bromoquinoline (Aldrich) using Zn dust and NiCl^2 bis(triphenylphosphine) in dimethylformamide (Sharma *et al.*, *J. Org. Chem. 59*:7785 (1995)).

Typically, the pharmacologically active substance is linked to the anilino derivative of the complex by the reaction with succinic anhydride to give the hemi-succinamide. The resulting hemi-succinamide may then be converted to an active ester with a dehydration agent, followed by reaction of the active ester with a nucleophilic functional group on the pharmacologically active substance. Examples of the active esters which may be used in the practice of the invention include the hemi-succinate esters of N-hydroxysuccinimide, sulfo-N-hydroxysuccinimide, hydroxybenzotriazole, and p-nitrophenol. Examples of dehydration agents include dicyclohexylcarbodiimide (DCC), 1-(3-dimethylaminopropyl)-3-

ethylcarbodiimide (EDC), and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide methiodide (EDCI).

Alternatively, where one of R^3, R^4, R^5 and R^6 is a halo group, a Grignard reagent may be prepared by reaction with magnesium, followed by condensation with a carbonyl-containing pharmacologically active substance. Examples of a pharmacologically active substances containing a carbonyl group are progesterone, rolipram, rolicyprine, and progabide.

Pharmacologically active substances which can be conjugated to the complexes of the present invention include, but are not limited to, enzymes, such as transferases, hydrolases, isomerases, proteases, ligases, kinases, and oxidoreductases such as esterases, phosphatases, glycosidases and peptidases; enzyme inhibitors such as leupeptin, chymostatin and pepstatin and growth factors such as tumor angiogenesis factor.

Other suitable pharmacologically active substances are fat-soluble steroids such as progesterone, estrogens and androgens, as well as the fat soluble vitamins A, D, E and K.

In addition to low and high molecular weight polypeptides, the pharmacologically active substance may be an anti-inflammatory agent (e.g., indomethacin, flurbiprofen, ketoprofen, ibuprofen, phenylbutazone), antibiotics (e.g., beta-lactams, aminoglycosides, macrolides, tetracyclines, pryridonecarboxylic acids, phosphomycin), anti-tumor agents (e.g., adriamycin, cisplatin, bleomycin, mitomycin, fluorouricil, vinblastine, vincristine), amino acids (e.g., ascorbic acid, N-acetyltryptophan), antifungal agents, prostaglandins, vitamins, steroids, and antiviral agents (AZT, DDI, acyclovir, idoxuridine, amantadine, and vidarabine).

The compositions according to the invention may be administered by means of the pharmaceutical or galenic formulations known and used by those skilled in the art for the particular method of administration, but preferably those used for parenteral administration, especially for intravenous, intramuscular, subcutaneous, intracutaneous, intraarticular, intrathecal, intraperitoneal infusion

or injection, including continuous infusions or intermittent infusions with the pumps available to those skilled in the art, or the administration by means of micro-encapsulated preparations, e.g., based on liposomes, e.g., according to EP-A-213,523.

For preparing a ready-to-use solution for the administration of metal complexes according to the invention, one may use the aqueous infusible and injectable solutions known for this purpose, optionally together with the excipients, carriers and/or stabilizing substances known in the art. A ready-to-use solution for the purposes of the invention may for example be prepared by dissolving the metal complex in water or in phosphate-buffered physiological saline solution (pH 7 to 7.5), optionally supplemented with Tween and/or gelatine or an albumin, before administration, the solution being transferred under sterile conditions into suitable containers (e.g., syringes, ampoules, bags).

The quantity of metal complex to be administered for the purposes of the invention will be determined in accordance with the dosages known in the art, the severity of the disease, the response rate and the further course of the disease and side effects. Generally speaking, the dosage must be adjusted according to individual criteria. Preferably, the metal complex is administered to an animal at an effective dosage level of from about 50 mg to 500 mg per day.

The method of administration and dosage will depend on the therapy plans known for the above-mentioned antimalarial agents, including also liposome-based microencapsulated antimalarial substances, e.g., according to EP-A-213,523 or EP-A-152,379 and also, for example, according to EP-A-354,442 or EP-B-56,781, to name just some of the numerous published patent literature.

The metal complexes according to the invention and conventional antimalarial substance can be administered either by simultaneous administration or by consecutive or sequential administration by suitable route, the individual active substances being provided and administered either separately, e.g., in the form of a kit or directly together. The active substance components which are

present separately or either indirectly or directly together may be provided both as dry substances and as solutions, while microencapsulated forms are also possible in which the active substance components may be used directly together, indirectly as a liposome mixture or as separate systems for administration. It is advantageous for the two active substance components, the antimalarial drug and metal complex, to be administered simultaneously.

It is intended that any animal may be treated with the pharmaceutical compositions of the present invention. Preferably, such animal is a human, however, the invention is not intended to be so limited.

Without further elaboration, it is believed that one skilled in the art can, using the preceding description, utilize the present invention to its fullest extent. The following preferred specific embodiments are, therefore, to be construed as merely illustrative, and not limitative of the remainder of the disclosure in any way whatsoever. All patents and publications cited herein are incorporated by reference herein in their entirety.

Example 1

Synthesis and Antimalarial Activity of ENBPI Complexes

Bioassays

Antimalarial Activity of Selected MENPBI-complexes

The potency of these novel metallopharmaceuticals to inhibit the intraerythrocytic growth of the malarial parasite in vitro was determined with the hypoxanthine incorporation method. Because growth of parasites in culture parallels incorporation of radiolabeled hypoxanthine into cellular DNA, antimalarial agents characteristically decrease hypoxanthine radioactivity found

within the cell pellets. For the following data, 100% inhibition represents complete eradication of the parasite.

Methods

Culture

Drug-sensitive *P. falciparum* clone HB3 and chloroquine-resistant *P. falciparum* clones FCR-3 and Indo were grown at 37°C under 3% oxygen/3% carbon dioxide in RPMI medium using 5% human red blood cells (Trager and Jensen, *Science 193*:673 (1976)) supplemented with 10% human plasma (Hui *et al.*, *Trans. R. Soc. Trop. Med. Hyg. 625*:625 (1984)). Synchronization was attained by treatment with sorbitol (Lambros and Vandenberg, *J. Parasitol. 65*:418 (1979)).

Drug Effects on P. falciparum Culture

Late ring-stage cultures at 10% parasitemia were grown in triplicate in the presence of various concentrations of MENPBI-complexes or MENPBA-complexes (diluted in RPMI medium) for 16 h. At the end of this period, 1μCi (17.2 Ci/mmol) of [^{3}H]hypoxanthine was added and the cultures were incubated for another 4h. Parasites were harvested and [^{3}H]hypoxanthine incorporation measured as previously described (Francis *et al.*, *EMBO J. 13*:306 (1994); Desjardins *et al.*, *Agents Chemother. 16*:710 (1979)). As a control, the DMSO vehicle for the drug was diluted in RPMI to the same extent and added to a similar culture. This had no effect on parasite hypoxanthine incorporation. Parasitemia in the cultures paralleled hypoxanthine incorporation.

For a time course, ring-stage cultures were incubated with or without addition of 10μM MENPBI-complexes for 6-21 h. At the designated times,

aliquots of cultured parasites were removed and blood smears prepared using Giemsa stain.

Isobologram Analysis

Late ring-stage cultures at 2% parasitemia were grown for 16 h in the presence of various combinations of the MENPBI-complexes (Francis *et al.*, *EMBO J.* *13*:306 (1994)) and the classic antimalarial agent, chloroquine. At the end of this period, 1 μCi (17.2 Ci/mmol) of [^{3}H]hypoxanthine was added and the cultures were incubated for another 4 h. Parasites were harvested and [^{3}H]hypoxanthine incorporation was measured as previously described (Desjardins *et al.*, *Agents Chemother.* *16*:710 (1979)). As a control, the DMSO vehicle for the drug was diluted in RPMI 1640 to the same extent and added to a similar culture. This had no effect on parasite hypoxanthine incorporation. Parasitemia in the cultures paralleled hypoxanthine incorporation. The IC_{50} for each inhibitor in the presence of sub-IC_{50} doses of the other inhibitor was calculated and plotted (isobologram).

Cell Survival Studies and LD_{50} Determinations

Survival of parental (KB-3-1) and multidrug-resistant (KB-8-5) cell lines exposed to MENPBI- or MENPBA-complexes was assayed in 96 well microtiter plates. Cells (4,000-20,000) were plated with increasing test concentrations of the novel cytotoxic agents (MENPBI- or MENPBA-complexes) and incubated for 72 h at 37°C in triplicate. Multidrug-resistant cells were cultured in drug free media for 72-96 hours prior to culture with cytotoxic agents. Cell survival was assayed using sulforhodamine B (SRB) (Mazzanti *et al.*, *J. Cell Pharmacol.* *1*:50 (1990)). Cells were fixed in 10% trichloroacetic acid for 60 minutes at 4°C, washed 5 times with tap water and stained with 0.4% SRB in 1% acetic acid for 15 minutes at room temperature. Excess SRB was removed with four 1% acetic

acid washes and stain redissolved in 10 mM unbuffered Tris base. Quantitation was carried out using an ELISA plate reader at a wavelength of 550 nm. Survival was expressed as the percentage of surviving cells relative to growth in MEM media without MENPBI- or MENPBA-complexes. LD_{50} determinations were obtained by interpolation from the cell survival curves.

Results

Figure 1 shows concentration-effect curves for the R^3-methoxy, R^3 ethoxy, or R^4,R^6-dimethoxy substituted MENPBI-Fe(III) complexes. All three monocationic Fe(III) complexes were potent inhibitors of the drug-sensitive HB3 strain of *P. falciparum*. Lineweaver-Burk plots of the inhibition curves revealed IC_{50} values (half-maximal inhibition concentrations) of 2.9 μM, 0.8 μM, and 1.1 μM, respectively. Surprisingly, the In(III) analog of the 4,6-dimethoxy MENPBI-complex showed only minimal antimalarial activity at all doses (data not shown).

A chemical structure-activity survey of the antimalarial action of these novel metallopharmaceuticals when present at concentrations of 1 μM and 5 μM was performed with drug-sensitive HB3 and two chloroquine-resistant *P. falciparum* clones, FCR-3 and Indo (Table 1). As in the previous experiment, 5 μM concentrations of all three MENPBI-Fe(III) complexes completely abrogated growth of the HB3 parasite. The FCR-3 clone showed reduced sensitivity to the antimalarial action of the 3-methoxy and 3-ethoxy MENPBI-Fe(HI) analogs, however, the 4,6-dimethoxy MENPBI-Fe(III) analog showed 96% growth inhibition at 5 μM. Surprisingly, with the Indo drug-resistant clone, all three MENPBI-Fe(III) complexes showed robust growth inhibition profiles at 5 μM. Again, the 4,6-dimethoxy MENPBI-In(III) analog was without significant activity in any clone. In view of the data set as a whole, the most important observation was that the 4,6-dimethoxy MENPBI-Fe(III) complex showed potent in vitro antimalarial activity in both drug-sensitive and chloroquine-resistant clones!

Table 1
Chemical Structure-Antimalarial Activity of Substituted Hexadentate MENBPI-Complexes Against Drug Sensitive (S) and Chloroquine-Resistant (CQR) *P. falciparum* Strains *In Vitro*

	Substitutions#				Strain		
Metal	R3	R4	R6	Conc (μM)	HB3 (S)	FCR (CQR)	Indo (CQR)
					% Growth Inhibition*		
Fe(III)	OMe	H	H	1	48.9	11	14.5
Fe(III)	OEth	H	H	1	22.5	34.8	23.2
Fe(III)	H	OMe	OMe	1	19.7	21.6	14.2
In(III)	H	OMe	OMe	1	NA	NA	NA
Fe(III)	OMe	H	H	5	90.4	31.1	51.6
Fe(III)	OEth	H	H	5	95.3	46.7	88.5
Fe(III)	H	OMe	OMe	5	99.9	96.1	96.1
In(III)	H	OMe	OMe	5	NA	NA	NA

NA = no antimalarial activity
* means of triplicate determinations
#R1, R5 = H

Additional data obtained in the same way shows that 5 μM of N,N'-bis[(3-(2-hydroxy-3-methoxybenzylamino)propyl]ethylenediamine]M(III)$^+$A$^-$and N,N'-bis[3-(2-hydroxy-3-methoxy-5-bromobenzylamino)propyl]-ethylenediamine M(III)$^+$A$^-$, where M is Fe or Al, completely inhibit the intraerythrocyte growth of the malarial parasite.

Figure 2 shows examples of concentration-effect curves for the R^4,R^6-dimethoxy substituted MENPBI-Fe(III) complex in a cytotoxicity assay with human KB renal cell carcinoma cells. The MENPBI-Fe(III) complex was a potent cytotoxic agent in the parental KB-3-1 cells demonstrating an IC$_{50}$ of approximately 8 μM. Interestingly, the derived multidrug-resistant KB-8-5 cells showed no evidence of cytotoxic effects at concentrations up to 100 μM,

indicating that expression of the human MDR1 P-glycoprotein transporter modulated the cytotoxic action of the MENPBI-complex.

Example 2

Antimalarial Schiff Base Fe(III) Coordination Complexes with Action Against Chloroquine-Resistant Plasmodium falciparum; Correlation with Inhibition of Hemozoin Formation

Introduction

Given the importance attributed to iron metabolites in parasite toxicity, a variety of metal chelating agents such as deferoxamine and reversed siderophores have been explored as potential antimalarial chemotherapeutics (Stahel, E. *et al., Amer. J. Trop. Med. Hygiene 39*:236-240 (1988); Yinnon, A. *et al., Blood 74*:2166-2171 (1989); Lytton, S. *et al., Mol. Pharma. 40*:584-590 (1991); Hershko, C. *et al., J. Inorg. Biochem. 47*:267-277 (1992); Gordeuk, V. *et al., Amer. J. Trop. Med. Hygiene 48*:193-197 (1993); Lytton, S. *et al., Amer. J. Hematology 43*:217-220 (1993)). When administered as free ligands, they have been shown to possess antimalarial activity, perhaps by disrupting ferric iron (Fe(III)) metabolism within the digestive vacuole, but none are ideal in their pharmacological properties (Hershko, C. *et al., J. Inorg. Biochem. 47*:267-277 (1992)). Other multidentate ligands, some containing an N_mO_n donor core (m,n = 2 to 4), have also been previously explored as therapeutic chelating agents for treatment of metal intoxication (May, P.M. and Bulman, R.A., *Prog. Med. Chem. 20*:225-336 (1983); Bryce-Smith, D., *Chem. Soc. Rev. 15*:93-123 (1986)), and their corresponding metal complexes evaluated as diagnostic radiopharmaceuticals (Deutsch, E. *et al., Prog. Inorg. Chem. 30*:75-139 (1983); Green, M.A. *et al., J. Am. Chem. Soc. 106*:3689-3691 (1984); Tsang, B. *et al., J. Nucl. Med. 34*:1127-1131 (1993); Tsang, B. *et al., J. Med. Chem. 37*:4400-4406

(1994)), as paramagnetic contrast agents in magnetic resonance imaging (Lauffer, R., *Chem. Rev. 87*:901-927 (1987); Kumar, K. and Tweedle, M.F., *Pure Appl. Chem. 65*:515 (1993); Xu, J. *et al., J. Am. Chem. Soc. 117*:7245-7246 (1995)), and as therapeutic medicinals (Abrams, M. and Murrer, B., *Science 261*:725-730 (1993)). We recently discovered that selected agents from one class, specifically hexadentate (N_4O_2) ethylenediamine-bis(propyl(R-benzylimino))Fe(III) complexes, are hydrolytically stable and possess the desired balance of hydrophobicity and delocalized monocationic charge that enhances cell membrane permeability (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). In mammalian cells, these compounds exhibit cytotoxic activity that is modulated by expression of the multidrug resistance (MDR1) P-glycoprotein (Pgp) in the plasma membranes (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). Human MDR1 P-glycoprotein is a homologue of the *P. falciparum* pfmdrl gene product, Pghl, both being integral membrane proteins of the ATP-binding cassette (ABC) superfamily of membrane transporters (Wilson, C. *et al., Science 244*:1184-1186 (1989); Foote, S.J. *et al., Nature 345*:255-258 (1990); Ford, J.M. and Hait, W.N., *Pharmacol. Rev. 42*:155-199 (1990); Gottesman, M.M. and Pastan, I., *Annual Review of Biochemistry 62*:385-427 (1993)).

While P-glycoprotein is an outwardly-directed drug transporter (Gottesman, M.M. and Pastan, I., *Annual Review of Biochemistry 62*:385-427 (1993); Piwnica-Worms, D. *et al., Biochemistry 34*:12210-12220 (1995)), Pghl has been localized to the digestive vacuole membrane (Cowman, A.F. *et al., J. Cell Biol. 113*:1033-1042 (1991)) and implicated in chloroquine sequestration (Krogstad, D.J. *et al., Biochem. Pharmacol. 43*:57-62 (1992)), possibly as an inwardly-directed transporter (van Es, H. *et al., Molec. and Cell Biol. 14*:2419-2428 (1994)). Pgh1 may contribute to chloroquine resistance in various clinical isolates of *P. falciparum* (Foote, S.J. *et al., Nature 345*:255-258 (1990)), although pfmdrl has been separated from chloroquine resistance in a genetic cross (Wellems, T.E., *et al., Nature 345*:253-255 (1990)).

Given the high degree of homology between the MDR1 P-glycoprotein and Pghl, we hypothesized that selected analogues of the N_4O_2 class of metal(III) compounds might be amenable to development as antimalarial chemotherapeutics. As potential antimalarial agents, these hexadentate Schiff-base metal complexes with the general structure according to formula *I* offer tremendous flexibility, since their metal binding affinities can be varied by inserting appropriate donor atoms to match the requirements of various coordinated metals, thereby enhancing stability of the intact complex (Wong, E. *et al., Inorg. Chem. 34*:93-101 (1995); Sharma, V. *et al., J. Chem. Med.*, submitted (1996)).

In addition, while maintaining the inner coordination sphere, the lipophilicity and molecular shape of the resulting complexes can be altered by variation of the substituents on the aromatic rings and hydrocarbon backbone independently. These compounds comprise a new class of readily synthesized antimalarials that, as chloroquine, block hemozoin formation, but additionally are active on chloroquine-resistant strains.

Materials and Methods

Synthesis of Ligands

All reagents, solvents and metal salts {nitrates and acetylacetonates of Al(III), Fe(III), Ga(III)), and In(III)} were obtained from Aldrich Chemical Co. or Alpha Chemical Co. as analytical grade materials. Heptadentate precursors of the desired hexadentate ligands **1a-b** according to Figure 6 were obtained by condensation of the appropriate linear tetramine and three equivalents of substituted salicylaldehyde in ethanol or in dry methylene chloride as previously described (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). 2-(2′-hydroxy-3′-methoxyphenyl)-1,3-bis(4-aza-5-(2″-hydroxy-3″-methoxy-phenyl)but-4′-ene-1′-yl)-1,3-imidazolidine (Mabi, 900 mg, 1.56 mmol) was synthesized by

condensation of N,N′-bis(aminopropyl) ethylenediamine (286 mg, 1.64 mmol) and o-vanillin (748 mg, 4.92 mmol) dissolved in ethanol. 2-(2′-hydroxy-4′,6′-dimethoxyphenyl)-1,3-bis(4-aza-5-(2″-hydroxy-4″,6″-dimethoxy-phenyl)but-4‴-en-1′-yl)-1,3-imidazolidine (Dmabi, 400 mg, 0.60 mmol) was obtained by a similar reaction of N,N′-bis(aminopropyl)ethylenediamine (130 mg, 0.74 mmol) and 4,6-dimethoxy-salicylaldehyde (407 mg, 2.24 mmol). Mabi and Dmabi were characterized by ^{1}H and ^{13}C NMR spectroscopy, infrared (IR) spectroscopy, mass (FAB) spectrometry (LR and HRMS) at the Washington University Resource for Biomedical and Bioorganic Mass Spectrometry Facility (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)).

Synthesis of N_4O_2 Metal(III) Complexes

Metal complexes were obtained through the reactions of Mabi and Dmabi with the appropriate metal salts in ethanol as described previously (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). Resulting metal(III) complexes were characterized by elemental analysis confirming purity and by ^{1}H NMR (except for Fe(III) complexes), IR, and FAB-LRMS establishing structural identity.

Plasmodium culture

Plasmodium falciparum strains (HB3, FCR-3, Indo-1, Dd2) were grown in intraerythrocytic culture by the method of Trager and Jensen (Trager, W. and Jensen, J.B., *Science 193*:673-675 (1976)). Cultures were maintained at 5% parasitemia, 2% hematocrit using human serum and erythrocytes, in a 3% oxygen/3% carbon dioxide atmosphere. Synchronization of developmental stage was achieved by sorbitol treatment (Lambros, C. and Vandenberg, J., *J. Parasitol. 65*:418-420 (1979)). Parasite growth inhibition and half-maximal inhibitory concentrations (IC_{50}) values were determined by measuring ^{3}H-hypoxanthine incorporation (Desjardins, R.E. *et al., Antimicrob. Agents Chemother. 16*:710-718

(1979)). Parasites were incubated with drug starting at the late ring stage and then ^{3}H-hypoxanthine added for 4 hours at the mid-trophozoite stage before harvesting parasites and assaying for incorporated radioactivity. All drugs were added as 1:1000 dilutions of a 10 mM DMSO stock. Vehicle alone had no effect on ^{3}H hypoxanthine incorporation.

Heme polymerization assay

The procedure used was modified from those of Slater & Cerami (*Nature (London) 355*:167-169 (1992)) and Dorn *et al.* (*Nature 374*:269-271 (1995)). Hemin (50 μM) was incubated with parasite-derived hemozoin (2 μg/ml) in 100 mM sodium acetate, pH 5.0 (final volume 0.5 ml). After 16 hours at 37°C, product was harvested by microfuge centrifugation at 4°C, 15,000 x g for 30 min. The pellet was resuspended by brief sonication in 1 ml of 2.5% sodium dodecyl sulfate (SDS) in 0.1 M sodium bicarbonate, pH 9.1, with incubation at 37°C for 30 min. Centrifugation was repeated and the resultant pellet washed in 1 ml of 2.5% SDS with sonication. After centrifugation, the pellet was solubilized with 2.5% SDS/50 mM NaOH (1 hr at room temperature with intermittent mixing). Product formation was measured spectrophotometrically at 400 nm wavelength. Control incubations had no added hemin or hemin alone and the sum of their values was subtracted. Individual inhibitors or chloroquine were added directly to the incubations, and were compared to incubations containing drug vehicle (DMSO), but without inhibitor. The histidine-rich protein II (HRP II)-mediated polymerization assay was performed as previously described (11-A) using 500 pmol protein incubated at 37°C for 16 h in a 1 ml reaction with 50 μM hematin.

Protease assays

The digestive vacuole proteases plasmepsins I and II and falcipain were purified and assayed using ^{14}C-globin substrate as previously described

(Gluzman, I. *et al., J. Clin. Invest. 93*:1602-1608 (1994)). Inhibitors were added as 1:1000 dilutions of a 10 mM DMSO stock.

Results

All reagents and starting materials were commercially available and inexpensive, favorable practical considerations for the development of these agents. The synthesis and characterization of these compounds are described elsewhere (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). An appealing property of the ethylenediamine-N,N′-bis(propyl(2-hydroxy-R-benzylimino)) ligands **1a-b** (R-ENBPI; Figure 6) is their ability to efficiently form stable "holo-complexes" with a wide range of metal ions (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). Specifically, we synthesized complexes of the general structure **2a-b** in Figure 3 coordinating Al(III), Fe(III), Ga(III) and In(III) from the reactions of Mabi and Dmabi precursors with corresponding metal(III) acetylacetonates in refluxing ethanol. Al(III) and In(III) complexes **2a-b** could also be isolated by treatment of precursors with their appropriate hydrated salts in the presence of base. Formation of metal complexes **2a-b** (Figure 3) with the ligands **1a-b** (Figure 6) and main group metal cations should be governed by the electronic and steric demands of the incoming metals relative to the flexibility of the donor core. In all cases, complexation of heptadentate precursors with the corresponding trivalent metal ions led to cleavage of the five-membered imidazolidine ring, thereby exposing the inner secondary amine nitrogens for coordination to the metals (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). This imparted some degree of pre-organization to the N_4O_2 donor core of **1a-b** to meet the demands of the incoming metal ion as the ligands wrapped around the coordination sphere. Note that coordination of metals with these ENBPI ligands results in formation of stereoisomers (Sarma, B.D. and Bailar, J.C., *J. Am. Chem. Soc. 77*:5476-5480 (1955)).

Because of the importance of Fe(III) in malarial metabolism and relative biocompatibility of this metal with the host, Fe(III) compounds were the target leads tested against *Plasmodium falciparum* trophozoites in intraerythrocytic culture. *P. falciparum* killing curves for the 4,6-dimethoxy-ENBPI Fe(III) derivative are shown in Figure 4A. IC_{50} values of 1 to 1.5 μM were obtained against chloroquine-sensitive HB3 as well as against resistant FCR-3, Indo-1, and Dd2 strains. Control experiments with chloroquine confirmed that the HB3 strain was sensitive, while the others were chloroquine-resistant (Table 2). While the 3-methoxy- ENBPI Fe(III) analog was equipotent against the drug-sensitive strain, in contrast to the 4,6-dimethoxy analog, the agent was less effective against the resistant FCR-3 and Indo-1 strains (May, P.M. and Bulman, R.A., *Prog. Med. Chem. 20*:225-336 (1983)).

Parasite death as measured by the ^{3}H-hypoxanthine incorporation assay correlated well with blood smear counts. The drug was effective at the mid-trophozoite stage; more mature parasites were resistant and less mature parasites grew normally until they developed into trophozoites, at which point they were killed by the treatment (Figure 4B). Parasites cultured in the presence of drug showed greatly diminished hemozoin formation. In contrast, control parasites matured normally, developing a large digestive vacuole filled with hemozoin pigment, and then undergoing normal schizogony.

We therefore assessed the ability of drug to block heme polymerization in an *in vitro* assay. Heme was incubated with pre-formed hemozoin under acidic conditions in the presence of varying concentrations of the 4,6-dimethoxy-ENBPI Fe(III) complex. Like chloroquine, this compound was a potent polymerization inhibitor, with an IC_{50} of approximately 4 μM (Figure 4A). Recent data suggest that the parasite's HRP II can initiate heme polymerization in the *Plasmodium* digestive vacuole (Sullivan, D. *et al., Science 271*:219-222 (1996)). The 4,6-dimethoxy- ENBPI Fe(III) complex showed equivalent potency in blocking polymerization in the HRP II assay (Figure 5).

To determine whether coordination of Fe(III) *per se* was critical for antimalarial activity or whether overall conformations of the intact metallopharmaceutical conferred the desired effect, culture and heme polymerization inhibition curves were also generated for 3-methoxy- and 4,6-dimethoxy-ENBPI complexes using a variety of coordinated metals (Table 2). An excellent correlation was found between inhibition of hemozoin formation and ability to kill the *Plasmodium* culture for all complexed metal species. Interestingly, the agents that were potent inhibitors of heme polymerization and *Plasmodium* culture were also the ones that have been shown to be recognized by the human MDR1 gene product, the P-glycoprotein efflux transporter (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)).

To further determine if demetallation reactions were potentially involved in inhibition of heme polymerization, a variety of metal salts were directly tested in the hemozoin assay (Table 4). Although ferric iron(III) and aluminum(III) cations were potent inhibitors of the process (IC_{50} values of 0.7 μM and 1.5 μM, respectively), gallium(III) had little effect (IC_{50} = 100 μM), despite the substantial antagonist activity of R-ENBPI gallium(III) complexes. Conversely, while the indium(III) cation showed modest inhibition (IC_{50} = 25 μM), the R-ENBPI indium(III) complexes were completely ineffective. With the exception of modest activity of iron(II), a variety of other physiological dicationic metals were ineffective (Table 4). Control experiments demonstrated that the counter anions (iodide, nitrate and chloride) were also without effect. Therefore, the lack of correlation between potency of metal(III) salts and their corresponding R-ENBPI metal(III) complexes for inhibition of heme polymerization again pointed toward the intact holo-complex as the pharmacologically active component.

Because of the association between R-ENBPI metal(III) complexes and P-glycoprotein transport, and since the agents appeared to target the digestive vacuole in *Plasmodium* culture, assays were performed to examine possible inhibition of vacuolar protease action. The 4,6-dimethoxy- ENBPI Fe(III) complex at 10 μM had no effect on the globin cleavage activities of aspartic

proteases plasmepsins I and II, nor of the cysteine protease falcipain (data not shown), each purified from cultured parasites.

Discussion

Disruption of hemozoin formation within the digestive vacuole requires that agents such as chloroquine, by mechanisms not fully characterized, be transported across several membrane bilayers, including the erythrocyte plasma membrane, the parasitophorous vacuolar membrane, the parasite plasma membrane and finally, the digestive vacuole membrane. Chloroquine is modestly lipophilic and possesses titratable protons that confer a net cationic charge in acid environments. Thus, it has been proposed that diffusive transmembrane transport and drug trapping may account for concentrative accumulation of chloroquine within the digestive vacuole (reviewed in Slater, A., *Pharmacol. Ther. 57*:203-235 (1993)). The net cationic charge provided by protonation of the agent or binding to high affinity sites within the vacuole (ferriprotoporphyrins) may prevent back-diffusion of the compound (Chou, A. *et al., Biochem. 19*:1543-1549 (1980)). Other contributions to chloroquine uptake and antimalarial action may involve its properties as an amphiphilic cation which enables adsorption onto phospholipid bilayers (Wernsdorfer, W. and McGregor, I., eds., *Malaria: Principles and Practice of Malariology*, Churchill Livingston, New York, p. 1818 (1988)) and, as with many hydrophobic compounds possessing a delocalized monocationic charge (Lichtstein, D. *et al., Proc. Natl. Acad. Sci. USA 76*:650-654 (1979); Chernoff, D.M. *et al., Biochim. Biophys. Acta 1147*:262-266 (1993)), may allow permeation across membranes and concentrative accumulation within cell interiors in response to the negative transmembrane potentials generated by living cells (Ritchie, R.J., *Prog. Biophys. Molec. Biol. 43*:1-32 (1984)). Additional non-physicochemical processes could also contribute, particularly parasitic expression of the pfmdr1 gene product, Pgh1, a member of the ABC superfamily of membrane transporters (Wilson, C. *et al., Science 244*:1184-1186 (1989); Foote,

S.J. *et al., Nature 345*:255-258 (1990); van Es, H. *et al., Molec. and Cell Biol. 14*:2419-2428 (1994)). In designing an innovative approach to antimalarial chemotherapy, we thought it desirable to exploit mechanisms of drug transport that may enable the agent to bypass chloroquine resistance pathways, but continue to target functions of the digestive vacuole, given the essential role and unique nature of this organelle to malarial organisms.

The present invention identifies a new class of antimalarial compounds with potent activity in *Plasmodium* culture, and significantly, against several chloroquine-resistant strains. Antimalarial potency in culture correlated with their ability to inhibit heme polymerization, the most potent lead being the 4,6-dimethoxy- ENBPI Fe(III) complex. Potency also correlated with the fold-drug resistance conferred by expression of MDR1 P-glycoprotein on cells exposed to these complexes, one measure of the ability of these agents to be transported by the human gene product (Sharma, V. *et al., J. Chem. Med.*, submitted (1996)). It is expected that accumulation of drug by the parasites is dependent on the *Plasmodium* P-glycoprotein homologue, Pgh-1, which is located on the digestive vacuole membrane (Cowman, A.F. *et al., J. Cell Biol. 113*:1033-1042 (1991)).

Interestingly, the same agents that are not recognized by the human P-glycoprotein, especially the R-ENBPI In(III) complexes, lacked significant parasitocidal activity in *Plasmodium* culture and did not inhibit hemozoin formation *in vitro*. It is unlikely that this can be attributed to differential demetallation reactions of the agents, since this study demonstrated poor correlation between metal(III) salts and intact metal(III) complexes for inhibition of hemozoin formation under acidic conditions and, furthermore, the agents have been documented by ^{1}H-NMR and UV/VIS spectroscopy to be hydrolytically stable at neutral pH (37°C) for 72 hrs (51-A). Molecular configuration may be relevant. Crystal structures of R-ENBPI Fe(III) and Ga(III) analogues show a trans configuration for the phenolic oxygens around the central coordination sphere (Ito, T. *et al., Chemistry Letters* 121-124 (1983); Tsang, B. *et al., J. Med. Chem. 37*:4400-4406 (1994)). However, preliminary molecular modeling data

suggest that the identity of the coordination metal in the central core has a profound influence on conformation of the molecule (Sharma, V. *et al., J. Nucl. Med.*, in press (1996)). Given the larger ionic radii of six-coordinated In(III) versus Fe(III) and Ga(III) (0.81 Å versus 0.65 Å and 0.62 Å, respectively) (Shannon, R.D., *Acta Cryst. A32*:751-767 (1976)), molecular modeling data suggest that steric constraints of the tetramine backbone favor the cis configuration for the R-ENBPI Indium(III) compounds (Sharma, V. *et al., J. Nucl. Med.*, in press (1996)). These studies must be extended to other ligands and metals and ultimately, crystal structures will be obtained to validate the modeling data. Nonetheless, compounds preferring the trans configuration are recognized by MDR1 P-glycoprotein, are able to block heme polymerization, and can produce parasite death, while those favoring the cis configuration cannot, potentially tying together these various biological processes into a common metabolic pathway. The data shows that confirmations of the intact holo-complexes confer pharmacological activity.

The IC_{50} values for culture inhibition are in some cases slightly lower than those for *in vitro* heme polymerization. Several possibilities could explain this observation. First, the conditions for heme polymerization in the digestive vacuole could be sufficiently different from those in the test tube to change the IC_{50} value. Second, the compounds could be accumulated against a concentration gradient in the digestive vacuole. Third, it is possible that blocking only a small amount of heme polymerization leads to build-up of enough toxic free heme to kill the intact parasites.

The R-ENBPI metal(III) complexes had equivalent potency in the pre-formed hemozoin-initiated polymerization assay as in the HRP II-mediated assay. These were similar to results obtained for chloroquine. A preferred model is that heme polymerization is initiated by HRP II-mediated bonding of hemes (Sullivan, D. *et al., Science 271*:219-222 (1996)). Once nucleation has occurred, polymerization can proceed non-enzymatically. Since both the R-ENBPI metal(III) complexes and chloroquine work on the hemozoin-seeded (protein-

free) as well as the protein-mediated reactions, this suggests that the blockade is either at the non-enzymatic polymer extension phase of the reaction, or a direct effect on the heme substrate.

The tetramine backbone can be varied and substituted in a variety of positions on the aromatic ring in this class of compounds. The agents are relatively easy and inexpensive to synthesize, which is a crucial feature for success of a useful antimalarial drug. In preliminary experiments, varying the ring substituents provided new compounds that are effective in the high nanomolar range. It is encouraging to have a group of agents with scaffolds incorporating a biologically compatible metal like Fe(III) that appear to intercept the same molecular target as chloroquine, but are not subject to the same resistance mechanisms.

Table Descriptions

Table 2 shows the effect of various R-ENBPI metal(III) complexes and chloroquine on intraerythrocytic *P. falciparum* growth in culture. The substituent nomenclature is referenced in Figures 3 and 6. Agents were tested at the indicated concentrations against chloroquine-sensitive and -resistant strains by the ^{3}H-hypoxanthine incorporation method; values are presented as percent growth inhibition relative to control cultures without drug. Data are presented as the mean of triplicate determinations from representative experiments. NAA, no antimalarial activity; $R^5 = H$.

Table 3 shows the IC_{50} values for inhibition of parasite growth and hemozoin formation that are tabulated from this study and compared to MDR1-mediated drug resistance values recalculated from data in Sharma, V. *et al., J. Chem. Med.*, submitted (1996). Derived from studies in human KB cells, the MDR1 data indicate the fold-resistance (IC_{50} in MDR cells/IC_{50} in drug-sensitive cells; Ford, J.M. and Hait, W.N., *Pharmacol. Rev. 42*:155-199 (1990)) that

expression of MDR1 P-glycoprotein conferred on the cytotoxic potency of the indicate metal(III) complexes.

Table 4 shows data for the inhibition of heme polymerization by metal salts. Heme polymerization was assayed by the pre-formed hemozoin nucleation reaction (see Methods) in the absence or presence of 10 μM concentrations of the indicated metal salts. Data are mean values of triplicate determinations from representative experiments.

Table 2
Antimalarial Activity of R-ENBPI Metal(III) Complexes Against Chloroquine-Sensitive (S) and -Resistant (CQR) *P. falciparum* Strains *in vitro*

	R-ENBPI Complexes				Strain		
Metal	Substitutions			Conc	HB3 (S)	FCR-3 (CQR)	Indo-1 (CQR)
	R^3	R^4	R^6	(μM)	% Growth Inhibition		
Fe (III)	OMe	H	H	5	90.4	31.1	51.6
Fe (III)	H	OMe	OMe	5	99.9	96.1	96.1
In (III)	H	OMe	OMe	5	NAA	NAA	NAA
		chloroquine		0.02	74.5	NAA	NAA

Table 3
Inhibition of *Plasmodium* Culture (HB3) and Hemozoin Formation by R-ENBPI Metal (III) Complexes Correlates with MDR1-Mediated Drug Resistance

ENBPI Ligand	Metal	Parasite Culture IC_{50} (μM)	Hemozoin IC_{50} (μM)	MDR1-Mediated Fold-Resistant
R = 3-MeO	Fe(III)	2	4	77
	Ga(III)	>20	50	2.6
	Al(III)	>20	30	2.6
	In(III)	>20	>50	1.4
R = 4,6-Di MeO	Fe(III)	1	4	>200
	Ga(III)	3	2	>200
	Al(III)	3	3	>200
	In(III)	>20	40	1.0

Table 4
Effect of Metal Salts (10 μM) on Heme Polymerization

Metal	% Inhibition of Heme Polymerization
Fe^{+2}	54
Fe^{+3}	100
Al^{+3}	93
In^{+3}	22
Ga^{+3}	0
Co^{+2}	0
Cu^{+2}	0
Ni^{+2}	0
Zn^{+2}	4
Ca^{+2}	0
Mn+2	2
Mg+2	0

Example 3

Effects of Multidrug Resistance (MDRI) P-glycoprotein Expression Levels and Coordination Metal on the Cytotoxic Potency of Multidentate (N_4O_2) Ethylenediamine-bis[propyl(R-benzylimino)]Metal(III) Cations

Introduction

Selected organic lipophilic monocations, exemplified by tetraphenyl phosphonium (Lichtshtein, D. *et al., Proc. Natl. Acad. Sci. USA 76*:650-654 (1979)), rhodamine 123 (Johnson, L. *et al., Proc. Natl. Acad. Sci. USA 77*:990-994 (1980); Davis, S. *et al., J. Biol. Chem. 260*:13844-13850 (1985)), and 1-methyl-4-phenylpyridinium (Langston, J.W. *et al., Science 219*:979-980 (1983); Ramsay, R.R. *et al., Biochem. Biophys. Res. Commun. 134*:743-748 (1986)), and selected organometallic lipophilic cations, exemplified by hexakis(2-methoxyisobutyl isonitrile)technetium(I) (Tc-SESTAMIBI) (Piwnica-Worms, D. *et al., Circ. 82*:1826-1838 (1990); Backus, M. *et al., Am. J. Physiol. Cell 265*:C178-C187 (1993); Crane, P. *et al., Europ. J. Nuc. Med. 20*:2025 (1993); Piwnica-Worms, D. *et al., Mag. Res. Imaging 12*:641-652 (1994)), accumulate within mitochondria of living cells. Characterized by modest hydrophobicity, enabling permeation of biological membrane bilayers, and a delocalized cationic charge, enabling sequestration into compartments generating negative transmembrane potentials, these agents concentrate up to 1,000-fold within the mitochondrial matrix. Exploiting this biological targeting property, the compounds have proven utility as biophysical probes of membrane function (Ritchie, R.J., *Prog. Biophys. Molec. Biol. 43*:1-32 (1984); Chernoff, D.M. *et al., Biochim Biophys Acta 1147*:262-266 (1993)), as models of neurological damage (Gluck, M. *et al., J. Biol. Chem. 269*:3167-3174 (1994)), and in medical imaging (Wackers, F.J. *et al., J. Nucl. Med. 30*:301-309 (1989)). Furthermore, a putative increase in mitochondrial transmembrane potential in cancer cells has been

proposed as a potential mechanism conferring tumor-specific cytotoxicity to selected lipophilic cations, such as rhodamine 123 and analogs (Davis, S. *et al.*, *J. Biol. Chem. 260*:13844-13850 (1985); Chen, L.B., *Annu. Rev. Cell Biol. 4*:155-181 (1988); Koya, K. *et al., Cancer Res. 56*:538-543 (1996)). Recently, carrier-added ^{99}Tc-SESTAMIBI was shown to possess potent cytotoxic activity against human Alexander hepatocellular carcinoma cells with a half-maximal lethal concentration (LC_{50}) of ~ 9 μM, putatively by disrupting mitochondrial function (Piwnica-Worms, D. *et al., Cancer Res. 53*:1-8 (1993)). However, regarding further development as therapeutic metallopharmaceuticals, ^{99}Tc(I) or isostructural rhenium(I) analogs are less than optimal because of the inherent low energy β-emissions and sluggish coordination reaction kinetics, respectively, of these metals. To further explore alternative scaffolds as potential targeted anticancer metallopharmaceuticals, we sought multidentate ligands capable of conveniently chelating a variety of metals, while retaining the biologically desirable lipophilic monocationic characteristics for conferring intracellular targeting.

A variety of multidentate ligands and their corresponding metal complexes have been explored as therapeutic chelating agents for the treatment of metal intoxication (Bryce-Smith, D., *Chem. Soc. Rev. 15*:93-123 (1986); May, P.M. and Bulman, R.A., *Prog. Med. Chem. 20*:225-336 (1983)), as diagnostic radiopharmaceuticals (Deutsch, E. *et al., Prog. Inorg. Chem. 30*:75-139 (1983)), as paramagnetic contrast agents in magnetic resonance imaging (Lauffer, R., *Chem. Rev. 87*:901-927 (1987); Kumar, K. and Tweedle, M.F., *Pure Appl. Chem. 65*:515 (1993); Xu, J. *et al., J. Am. Chem. Soc. 117*:7245-7246 (1995)), as therapeutic medicinals (Abrams, M. and Murrer, B., *Science 261*:725-730 (1993)), to perform synthetic transformations (e.g., asymmetric epoxidations of unfunctionalized olefins (Palucki, M. *et al., Tetrahedron Lett. 36*:5457-5460 (1995)), and to study guest-host interactions of various ligands and metals.

Among multidentate ligands, Schiff-base ligands (Isobe, T. *et al., Bull. Chem. Soc. Japan 40*:1862-1863 (1967); Bailey, N.A. *et al., Inorg. Chim. Acta*

25:L137-138 (1977); Evans, D.F. and Jakubovic, D.A., *J. Chem. Soc. Dalton Trans.* 2927-2933 (1988)) with an N_4O_2 donor core are well known. The coordination chemistry of these ligands with various transition and main group metals (Sarma, B.D. and Bailar, J.C., *J. Am. Chem. Soc.* *77*:5476-5480 (1955); Sarma, B.D. *et al.*, *J. Am. Chem. Soc.* *86*:14-16 (1964); Tweedle, M.F. and Wilson, L.J., *J. Am. Chem. Soc.* *98*:4824-4834 (1976)) and structural aspects of various metal chelates having N_4O_2 and N_4O_3 donor cores with main group metals and lanthanides have been reported (Wong, E. *et al.*, *Inorg. Chem.* *34*:93-101 (1995); Yang, L.W. *et al.*, *Inorg. Chem.* *34*:2164-2178 (1995); Yang, L.W. *et al.*, *Inorg. Chem.* *34*:4921-4925 (1995)).

Schiff-base N_4O_2 metal complexes have also been reported as potential positron-emitting radiopharmaceuticals for use in myocardial perfusion imaging (Green, M.A. *et al.*, *J. Am. Chem. Soc.* *106*:3689-3691 (1984); Tsang, B.W. *et al.*, *J. Nucl. Med.* *34*:1127-1131 (1993); Tsang, B.W. *et al.*, *J. Med. Chem.* *37*:4400-4406 (1994)).

As potential cytotoxic agents, these hexadentate Schiff-base ligands with the general structure shown in Figure 6, or according to formula ***I*** offer tremendous flexibility, since their binding affinities can be varied by inserting appropriate donor atoms to match the requirements of various incoming metals. In addition, while maintaining the inner coordination sphere, the lipophilicity and molecular shape of the resulting complexes can be altered by variation of the substituents on the aromatic rings and hydrocarbon backbone independently.

The synthesis of N_4O_2 Schiff base phenolic complexes of Al(III), Fe(III), Ga(III), and In(III) are provided, as well as their characterization of their cytotoxic potency in human epidermal carcinoma KB-3-1 cells and the colchicine-selected multidrug resistant derivative KB-8-5 cells. For these Schiff base ligands, the cytotoxic potency of their corresponding metallopharmaceuticals depended strongly on the identity of the coordinating central metal. Furthermore, modest expression of the human multidrug resistance

(*MDR1*) gene product, P-glycoprotein, conferred protection from the cytotoxic activity of potent A1(III)-, Fe(III)- and Ga(III)-complexes.

Results and Discussion

Chemistry

Heptadentate compounds **3** and **4** precursors (Figure 7) of the desired hexadentate ligands, were synthesized by condensation of the appropriate amine and three equivalents of substituted salicylaldehyde in ethanol (Figure 7). Alternatively, condensation of the amine with three equivalents of substituted salicylaldehyde in dry methylene chloride in the presence of activated molecular sieves provided spectroscopically identical compounds. Infrared spectra of precursors **3** (**H_3Mabi**) and **4** (**H_3DMabi**) (Figure 7) showed strong peaks at 1640 cm^{-1} and 1625 cm^{-1}, respectively, characteristic of imine C=N bonds. Formation of a five membered imidazolidine ring due to reaction of a third aldehyde with the two inner adjacent secondary amine nitrogens of N,N'-bis(aminopropyl)ethylenediamine conferred unique characteristics on the middle ring compared to the two outer aromatic rings. The ^{1}H NMR spectrum of **3** in $CDCl_3$ showed the characteristic imine proton at δ 8.12, multiplets for aromatic protons at δ 7.00-6.55, methoxy substituents at δ 3.88 and δ 3.86 for the outer and middle rings, respectively, and hydrocarbon protons in the form of overlapping multiplets at δ 3.65, 3.40, 2.80-2.28, and 1.82. Proton-decoupled ^{13}C NMR spectrum showed 20 resonance signals (Figure 8), including the characteristic carbon resonance at δ 165.2 for the imine carbon and δ 89.3 for the benzylic carbon, consistent with the proposed structure for precursor **3**. The ^{1}H NMR of precursor **4** in $CDCl_3$ also showed the characteristic imine proton singlet at δ 8.25, aromatic protons at δ 5.99, 5.91, 5.84, and 5.56 in a ratio of 1:1:2:2, for the middle and outer aromatic rings, respectively, a proton assigned to the benzylic position at δ 4.32, different methoxy groups at δ 3.77, 3.76, 3.71, and

3.70 in a ratio of 2:2:1:1, respectively, and protons for the hydrocarbon backbone in the form of a series of multiplets at δ 3.52, 3.35, 2.75-2.30, and 1.80. Proton-decoupled ^{13}C NMR spectrum of precursor **4** in $CDCl_3$ showed 20 resonance signals instead of 22 (Figure 8), presumably because of overlapping methoxy signals.

When treated with trivalent metals in aqueous conditions, the heptadentate precursors, whether isolated as **3** and **4** or formed as Schiff base byproducts during condensation reactions of appropriate salicylaldehydes and amines, split off the middle ring, thereby decreasing steric demands on the resultant ligands and providing an N_4O_2 hexadentate donor core (Sarma, B.D. and Bailar, J.C., *J. Am. Chem. Soc. 77*:5476-5480 (1955)). An appealing property of these ligands (R-ENBPI; Figure 6) that we hoped to exploit was their ability to efficiently chelate a wide range of metal ions. Specifically, we synthesized complexes with Fe(III), Ga(III) and In(III) from the reactions of precursors **3** and **4** with corresponding metal(III) acetylacetonates in refluxing ethanol (Figure 7). Alternatively, Al(III) and In(III) complexes were isolated by treatment of precursors **3** and **4** with their appropriate hydrated salts in the presence of base. Formation of metal complexes with the ligands (Figure 6) derived from precursors **3** and **4** and main group metal cations should be governed by the electronic and steric demands of the incoming metals relative to the flexibility of the donor core. Due to the nearly identical six-coordinate ionic radii of Fe^{+3} and Ga^{+3} (0.65Å and 0.62Å, respectively (Shannon, R.D., *Acta Cryst. A32*:751-767 (1976))), ligands with high affinity for Fe^{+3} tend to have high affinity for Ga^{+3}, often resulting in similar coordination chemistry. In contrast, $A1^{+3}$ (0.51Å)and In^{+3} (0.81Å) were selected as two extreme limits to analyze the impact of the central metal core on the organic scaffold and to evaluate their overall effect on biological activity. In all cases, complexation of heptadentate compounds **3** and **4** with their corresponding trivalent metal ions lead to cleavage of the five-membered imidazolidine ring, thereby exposing the inner secondary amine nitrogens for coordination to the metals. This imparted some degree of pre-

organization to the N_4O_2 donor core to meet the demands of the incoming metal ion as the ligands wrapped around the coordination sphere. The ^1H NMR spectra of metal(III) complexes recorded in dimethylsulfoxide-d^6 revealed the absence of benzylic protons and relative simplicity in the aromatic proton region. Because synthesis of the complexes was carried out under aqueous conditions, their ^1H NMR spectra in dry dimethylsulfoxide-d^6 all showed a strong signal around δ 3.25-3.45 tentatively assigned to weakly coordinated water due to hydrogen bonding with the metal complexes. To test this hypothesis; exchange experiments were performed by adding traces of D^2O which produced disappearance of this signal, thereby confirming the presence of coordinated water and, furthermore, allowing proton signals arising from the hydrocarbon backbone underneath this signal to be assigned. Additionally, as expected, proton signals attributed to N-H around δ 4.80 - 5.22 either disappeared or broadened and moved upfield, whereas the rest of the signals remained unaltered in the D^2O exchange experiment.

Infrared (IR) spectra as KBr pellets of A1(III), Fe(III), Ga(III), and In(III) metal complexes were essentially identical, with only marginal differences in a few selected bands. Broad bands between 3250-3100 cm^{-1} and 1550-1560 cm^{-1} were assigned to stretching and bending modes, respectively, of the coordinated amine nitrogens. All complexes, except **3b**, showed a broad band between 3600-3300 cm^{-1} which we attributed to O-H stretchings in the metal complexes, suggestive of weakly coordinated water held through hydrogen bonding.

In addition, a characteristic band corresponding to υ(C=N) underwent a shift of 5-20 cm^{-1}, confirming coordination of the metals to the imine nitrogens of the ligands. Furthermore, normal bands at 3000-2600 cm^{-1}, and at 1600 cm^{-1}, 1480 cm^{-1}, and 1440 cm^{-1} were observed and assigned to υ(C-H) and υ(C=C), respectively. The appearance of bands below 600 cm^{-1} present in the spectra of complexes, but not in those of the precursors themselves, was probably indicative of υ(M-O) and υ(M-N).

Thus, the ^{1}H NMR and IR spectral data were consistent with the proposed structures of the intact complexes in solution and in solid state, respectively. Mass spectra (FAB) of all complexes done as 3-nitrobenzyl alcohol or thioglycerol matrices demonstrated mass ion peaks corresponding to the metal ligand cations ($(ML)^+$, M=metal, L=ligand). Furthermore, absence of peaks in the region of higher atomic mass indicated the absence of dimeric species and in turn supported the proposed monomeric cationic complexes.

Molar conductance (κ) measurements for the complexes in acetonitrile at room temperature showed values in the range of 110-174 Ω^{-1} mol^{-1} cm^2, also consistent with a 1:1 electrolyte (monocationic metal complexation) (Geary, W.J., *Coord. Chem. Rev. 7*:81-122 (1971)). Furthermore, complexes were stable to neutral hydrolysis. ^{1}H NMR and UV/VIS spectra of complexes were superimposible before and after incubation in water for 72 hr (37°C; pH 7.5).

The R-ENBPI derivatives of precursors **3** and **4** (Figure 7), containing an N_4O_2 donor core with the general structure shown in Figure 6, wrapped around the central metal ion to provide four six-membered rings and one five-membered ring. In contrast, two six-membered and three five-membered rings are created by compounds containing bis ethylene groups (Sarma, B.D. and Bailar, J.C., *J. Am. Chem. Soc. 77*:5476-5480 (1955)) instead of bis propylene groups. As a result of the steric requirements of the hydrocarbon chain, the propylene backbone links adjacent donor atoms so that they must be placed cis to each other, therefore generating angles between adjacent donor atoms close to 90°. This has been reported earlier in the case of {1,10-bis(2-hydroxybenzamido)-3,6-diazadecane}manganese(IV) (Chandra, S.K. and Chakravorty, A., *Inorg. Chem. 31*:760-765 (1992)).

Based upon the crystal structures of analogous R-ENBPI complexes of Fe(III) and Ga(III) (Tsang, B.W. *et al., J. Med. Chem. 37*:4400-4406 (1994); Ito, T. *et al., Chemistry Letter* 121-124 (1983)), we propose that our R-ENBPI complexes of A1(III), Fe(III), and Ga(III) likely have structures in which the two phenolate oxygens are coordinated trans to one another and cis to the

corresponding amine and imine nitrogens, yielding an overall octahedral geometry as shown in Figure 7 or the corresponding enantiomers. However, in the case of the In(III) complexes, the increase in ionic size obtained by moving down the periodic table within the main group metals would likely lead to increased steric constraints on the hydrocarbon backbone.

Therefore, it is expected that upon coordination of In(III) a significant change in structure to overcome these constraints, perhaps to a cis arrangement for the phenolic oxygens, may occur. Indeed, preliminary molecular modeling data using the CAChe worksystem correctly predicts the trans arrangement of phenolic oxygens around the central metal core for R-ENBPI A1(III), Fe(III) and Ga(III) complexes, but suggest the cis configuration for In(III) complexes (Sharma, V. *et al., J. Nucl. Med.*, submitted (1996)).

Cytotoxicity

P-glycoprotein, encoded by the human multidrug resistance (*MDR1*) gene, is an integral plasma membrane transporter which renders tumors resistant to chemotherapy by transporting chemotherapeutic agents out of cells (Gros, P. *et al., Nature 323*:728-731 (1986); Shen, D.W. *et al., Science 232*:643-645 (1986); Gottesman, M.M. and Pastan, I., *Annual Review of Biochemistry 62*:385-427 (1993)). Overexpression of P-glycoprotein is thought to be one potent mechanism of cancer chemotherapeutic failure in patients. Many drug substrates and modulators (inhibitors) of P-glycoprotein are lipophilic cationic compounds (Ford, J.M. *et al., Pharmacol. Rev. 42*:155-199 (1990)), suggesting that the relationship between cellular cytotoxicity of racemic mixtures of the R-ENBPI metal(III) complexes and expression levels of P-glycoprotein was important to explore. Parental human epidermal carcinoma KB-3-1 cells and the derivative colchicine-selected KB-8-5 multidrug resistant cells (Akiyama, S.I. *et al., Somatic Cell Mol. Genet. 11*:117-126 (1985)) express no immunodetectable and modest levels of *MDR1* P-glycoprotein, respectively, as determined by

immunoblots of plasma membrane preparations with the anti-P-glycoprotein monoclonal antibodies C219 (Dolci, E.D. *et al., International Journal of Cancer* *54*:302-308 (1993)) and C494.

These cells therefore provided a convenient *in vitro* system for quantitative cytotoxicity assays. Cells in monolayer culture in 96-well plates were exposed to novel R-ENBPI metal-complexes over a range of pharmacologically relevant concentrations and cell survival was determined by the SRB method after three days in culture (Skehan, P. *et al., J. Natl. Cancer Inst.* *82*:1107-1112 (1990)). Cells grown in the presence of drug vehicle alone served as control preparations, while cells grown in the presence of high concentrations of the chemotherapeutic agent colchicine (25 μM) documented the effects of maximal cytotoxic activity.

Cytotoxic potency was determined by computer fitting of survival curves and determination of an LC_{50}. In drug-sensitive KB-3-1 cells, (3-MeO-R-ENBPI)Fe(III) (**3b**, see Experimental) demonstrated an LC_{50} of 9 μM, the most potent cytotoxic activity for the 3-MeO-R-ENBPI series (Figure 9A-D). Metal(III) complexes of 3-MeO-R-ENBPI showed a rank order cytotoxic potency of Fe(III) > A1(III) > In(III) > Ga(III) (Table 5). Expression of *MDRI* P-glycoprotein by the KB-8-5 tumor cells conferred dramatic protection from the cytotoxic action of (3-MeO-R-ENBPI)Fe(III) (**3b**), with concentrations of **3b** as high as 100 μM demonstrating no significant cytotoxicity. Additionally, *MDRI* P-glycoprotein conferred modest protection (2-3 fold) from the cytotoxic action of **3a** and **3c**, but conferred no significant protection from **3d**. Similarly, in KB-3-1 cells, metal(III) complexes of 4,6-diMeO-R-ENBPI showed a rank order cytotoxic potency of Fe(III) > A1(III) > Ga(III) >> In(III) (Figure 9E-H, and Table 5).

Overall, the 4,6-diMeO analogs were more potent than the 3-MeO analogs. Again, expression of modest levels of *MDRI* P-glycoprotein in the KB-8-5 cells conferred robust protection from the cytotoxic action of the (4,6-diMeO-R-ENBPI)Fe(III) analog (**4b**) and, in this case, also from (4,6-diMeO-R-

ENBPI)A1(III) (**4a**) and from (4,6-diMeO-R-ENBPI)Ga(III) (**4c**), but conferred no significant modulation of the activity of the In(III) analog **4d**. These data would be most consistent with A1(III), Fe(III), and Ga(III) complexes being recognized as transport substrates by the human *MDRI* P-glycoprotein and thereby, extruded from the cells and sequestered away from their cytotoxic targets.

Interestingly, there appeared to be a correlation between cytotoxic potency and P-glycoprotein transport activity. As a group, the active 4,6-diMeO-R-ENBPI analogs were more potent and showed a more robust P-glycoprotein transport profile than the 3-MeO-R-ENBPI analogs. In(III) complexes **3d** and **4d** were conspicuously low in cytotoxic potency and devoid of a P-glycoprotein transport profile. In addition, cytotoxic activities of nitrate salts of A1(III), Fe(III), Ga(III) and In(III) were evaluated in KB-3-1 and drug-resistant KB-8-5 cells (Figure 9I-L).

In contrast to their corresponding R-ENBPI complexes, A1(III) and Fe(III) ions did not show any evidence of cytotoxicity at any concentration up to 100 μM or any differential effect between drug-sensitive and MDR cells. Ga(III) and In(III) salts demonstrated modest toxic activity at 100 μM with a trend towards increased potency in the drug-resistant cell line, opposite to effects observed with the metal complexes. Furthermore, precursors **3** and **4**, when added to the aqueous buffer (thereby likely providing the free hexadentate ligand) demonstrated no significant cytotoxic activity except at the highest test concentration of 100 μM where modest activity was observed equally in both cell lines (data not shown). Overall, when combined with the demonstrated stability of the complexes to neutral hydrolysis, these data indicated that the cytotoxic potency of the A1(III), Fe(III) and Ga(III) complexes would best be attributed to pharmacological effects of the intact complex *per se* within cellular compartments.

The observation that A1(III), Fe(III) and Ga(III), but not In(III) complexes provided the desired cytotoxic properties further argued that the proposed

molecular shape (trans phenolic configuration) and charge distribution of the final complexes, not metal-specific properties, generally conferred the pharmacological actions. The stability of the complexes to neutral hydrolysis and lack of activity of the metal(III) ions would tend to exclude rapid extracellular demetallation reactions, transmetallation reactions with serum metal-binding proteins such as transferrin, or chelation reactions of ligands in neutral intracellular compartments as contributors to their mechanism of action. It would appear that coordination of In(III) distorted or destabilized the complex sufficiently to alter its capacity to accumulate at its cytotoxic target (putative electronegative compartments) or to be recognized by the MDRI transporter.

It is expected that the preferential potency of the Fe(III)-complexes may be attributable to specific conformational dimensions of the complex, but cannot exclude the possibility of acid hydrolysis of these agents within subcellular organelles such as lysosomes. While these metallopharmaceuticals were designed to exploit electronegative compartments such as mitochondria as a cytotoxic target, further experiments are required to explore the pharmacological sites, mechanism(s) of action, stereochemistry of drug activity and impact of co-administration of MDR modulators on their anticancer potency.

Conclusions

Monocationic A1(III), Fe(III), Ga(III), and In(III) complexes of substituted Schiff base N_4O_2 phenol ligands (R-ENBPI) were synthesized and their pharmacological properties explored as a potential class of cytotoxic metallopharmaceuticals. These complexes combine linear ligand flexibility enabling the coordination of a variety of core metals with the lipophilic cationic characteristics of potential-dependent tumor-selective pharmaceuticals. Results showed that the cytotoxic potency of racemic mixtures of these metallopharmaceuticals depended strongly on the identity of the coordinating central metal in the rank order Fe(III) > A1(III) > Ga(III) $\geq$ In(III). In addition,

the active 4,6-dimethoxy substituted R-ENBPI complexes were more potent than the 3-methoxy analogs.

Furthermore, modest cellular expression of the human *MDRI* P-glycoprotein conferred protection from the cytotoxic activity of A1(III), Fe(III) and Ga(III) R-ENBPI complexes, but not In(III) complexes. Remarkably, among the Group(III) elements, human cells were thus capable of distinguishing complexes formed of the same ligands with different metals. A trans configuration of the phenolic moieties around the central coordination sphere of A1(III), Fe(III) and Ga(III) R-ENBPI complexes along with differential stabilities as compared with In(III) complexes may impart these biological activities. However, further studies are needed to evaluate mechanism(s) of action and structure-function relationships of these cytotoxic metal complexes.

Experimental Section

General Methods

Bis(N,N'-aminopropyl)ethylenediamine, 4,6-dimethoxy salicylaldehyde and O-vanillin (3-methoxy salicylaldehyde) were obtained from Aldrich Chemical Co. A1(III)-, Fe(III)-, Ga(III)-, and In(III)-acetylacetonates and their hydrated salts were purchased from Mathey-Johnson/Alfa Chemical Co. and Aldrich. The ^{1}H and ^{13}C NMR spectra were recorded on a GEMINI 300 HHz spectrometer; chemical shifts are reported in δ (ppm) with reference to TMS. IR spectra were recorded on a Perkin-Elmer 1710 Fourier transform spectrophotometer. Mass spectra (LR and HRMS) were obtained from the Washington University Resource for Biomedical and Bioorganic Mass Spectrometry with 3-nitrobenzyl alcohol or thioglycerol as a matrix. Elemental analyses (C, H, N) were performed by Galbraith Laboratories, Knoxville, TN. Molar conductance (κ, Ω^{-1} mol^{-1} cm^{2}) was determined with a portable conductivity meter (Orion Research, model 120) at 25°C in acetonitrile with 0.37

mM solutions of each complex. Aqueous stabilities of the complexes were determined by suspending 5-10 mg of sample in 2 ml of H_2O (pH 7.5) in a sealed vial. Samples were stirred for 72 h (37°C), evaporated and residue analyzed by UV/VIS (Beckman 620) or ^{1}H NMR spectroscopy in DMSO-d^6.

Synthesis of Ligands

2-(2′-hydroxy-3′-methoxyphenyl)-1,3-bis (4-aza-5- (2″-hydroxy-3″-methoxyphenyl)but-4′-ene-l′-yl)-1,3-imidazolidine (H_3Mabi) (3):

N,N'-bis(aminopropyl)ethylenediamine (286 mg, 1.64 mmol) dissolved in ethanol (5 ml) was added to a stirred solution of o-vanillin (748 mg, 4.92 mmol) dissolved in ethanol (10 ml). The reaction mixture was heated to reflux for 3 h. After cooling to room temperature, volatiles were removed through rotatory evaporation and the residue dried under reduced pressure for two days to yield a bright yellow solid **1** (900 mg, 1.56 mmol, 95% yield). Alternatively, treatment of N,N'-bis(aminopropyl)ethylenediamine with three equivalents of o-vanillin in dry methylene chloride in the presence of activated molecular sieves at room temperature stirred for overnight and filtration followed by its subsequent evaporation gave a spectroscopically identical compound. ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.82 (m, 4H), 2.28-2.80 (m, 6H), 3.40 (m, 4H), 3.65 (m, 3H), 3.86 (s, 3H), 3.88 (s, 6H), 6.55 (dd, 1H), 6.64 (t, 1H), 6.7-7.0 (m, 7H), 8.12 (s, 2H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ: 28.7, 49.4, 49.6, 55.6, 55.7, 55.8, 89.3, 111.9, 113.4, 117.1, 117.9, 118.1, 121.2, 122.7, 122.8, 147.2, 147.7, 148.3, 152.4, 165.2; IR (CH_2C1^2, cm^{-1}): 1640 υ(C=N); FAB-HRMS: calculated for $C_{32}H_{41}N_4O_6$ $(M+H)^+$ 577.3025, found 577.3020.

2-(2′-hydroxy-4′,6′-dimethoxyphenyl)-1,3-bis (4-aza-5- (2″ -hydroxy -4″,6″-dimethoxyphenyl)but-4‴-en-1′-yl)-1,3-imidazolidine (H_3DMabi) (4):

A similar reaction of N,N'-bis(aminopropyl)ethylenediamine (130 mg, 0.74 mmol) and 4,6-dimethoxy salicylaldehyde (407 mg, 2.24 mmol) produced a pale yellow solid H_3DMabi **2** (400 mg, 0.60 mmol, 80%). ^{1}H NMR (300 MHz, $CDCl_3$) δ: 1.80 (m, 4H), 2.30-2.75 (m, 6H), 3.35 (m, 4H), 3.52 (m, 2H), 3.70, 3.71 (s, 6H), 3.77, 3.76 (s, 12H), 4.32 (s, 1H), 5.56 (d, 2H), 5.84 (d, 2H), 5.91 (d, 1H), 5.99 (d, 1H), 8.25 (s, 2H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ: 28.9, 49.2, 49.5, 50.8, 54.9, 55.1, 80.6, 87.2, 89.4, 94.2, 95.2, 101.1, 102.0, 158.1, 160.1, 160.5, 160.6, 161.4, 166.8, 176.0; IR (CH_2C1^2, cm^{-1}): 1625, υ(C=N); FAB-HRMS: calcd for $C_{35}H_{47}N_4O_9$ $(M+H)^+$667.3342, found 667.3338.

Synthesis of Metal(III) Complexes

Method A:

Appropriate ligand was dissolved in methanol (3 ml), treated dropwise with potassium hydroxide and heated to reflux for 20 min. While hot, the appropriate hydrated salt dissolved in methanol (5 ml) containing water (2ml) was added, refluxed for 1 h, cooled and filtered. The filtrate was slowly evaporated at room temperature to yield a microcrystalline solid, which was separated, washed with water, followed by cold methanol, then washed with ether and dried under reduced pressure overnight.

Method B:

Another method involved mixing equimolar quantities of the appropriate ligand and metal acetylacetonates in ethanol. Contents were refluxed for 1 h, then the reaction mixture was treated while hot with an equimolar amount of K1 dissolved in water, and then refluxed for an additional 5-10min. The resulting mixture was cooled to room temperature and a microcrystalline compound

precipitated out. This was separated, washed with cold ethanol, then washed with ether and dried under reduced pressure.

((3-OMe-R-ENBPI)A1)$^+$NO$_3^-$(3a)

Using Method A involving H_3Mabi ligand **3** (160 mg, 0.27 mmol), potassium hydroxide(271 μl, 3M), and A1$(NO_3)^3 \cdot 9H_2O$(100 mg, 0.27 mmol) and washings with ether containing 10% MeOH and finally with ether and drying under vacuum provided **3a** (130 mg, 0.24 mmol, 87%). ^{1}H NMR (300 MHz, DMSO-d^6) δ: 1.50 (m, 2H), 1.85 (m, 2H), 2.68 (m, 2H), 2.88 (m, 4H), 3.15 (m, 2H), 3.38 (m, 4H), 3.68 (s, 6H, OCH_3), 4.90 (bs, 2H, NH), 6.65 (t, 2H, Ar-H), 6.90 (d, 2H, Ar-H), 7.05 (d, 2H, Ar-H), 8.30 (s, 2H, CH=N); IR (KBr, cm^{-1}): 1625 υ (C=N), 1360, 1265 υ(NO_{3-}); κ(Ω^{-1} mol^{-1} cm^2) 117 ; Mass Spectrum (FAB) for $(C_{24}H_{32}N_4O_4Al)^+$ m/z = 467.1; Anal. calcd for $C_{24}H_{32}N_5$07Al · H_2O: C, 52.65; H, 6.26; N, 12.79 found: C, 52.50; H, 6.22; N, 12.69.

((4,6-di-OMe-R-ENBPI)Al)$^+$NO$_3^-$ (4a)

Using Method A involving H_3DMabi ligand **4** (66 mg, 0.09 mmol), potassium hydroxide (3 M, 98 μl) and A1$(NO_3)^3 \cdot 9H_2O$ (37 mg, 0.09 mmol) yielded **4a** (50 mg, 0.08 mmol, 86 %). ^{1}H NMR (300 MHz, DMSO-d^6) δ: 1.50 (m, 2H), 1.90 (m, 2H), 2.70 (m, 2H), 2.92 (m, 4H), 3.15- 3.65 (m, 6H), 3.78 (s, 6H, OCH_3), 3.81 (s, 6H, OCH_3), 4.80 (bs, 2H, NH), 5.94 (s, 2H, Ar-H), 6.02 (s, 2H, Ar-H), 8.22 (s, 2H, CH=N); IR (KBr, cm^{-1}): 1620 υ(C=N), 1280b υ(NO_{3-}); κ(Ω^{-1} mol^{-1} cm^2): 158; Mass Spectrum (FAB) for $(C_{26}H_{36}N_4O_6A1)^+$ m/z = 527.2; Anal. calcd for $C_{26}H_{36}N_5O_9Al \cdot H_2O$: C, 51.40; H, 6.30; N, 11.53 found: C, 51.61; H, 6.40; N, 11.65.

((3-OMe-R-ENBPI)Fe)$^+$I$^-$ (3b)

Using Method B involving H_3Mabi ligand **3** (153 mg, 0.26 mmol) and Fe(acac)^3 (94 mg, 0.26 mmol) and KI (44 mg) resulted in yield of **3b** (120 mg, 0.19 mmol, 72%). IR (KBr, cm^{-1}): 3140 υ(N-H), 3000-2750 υ(C-H), 1620

υ(C=N), 1555 δ(N-H), 1605, 1480, 1440 υ(C=C); $\kappa(\Omega^{-1}\ mol^{-1}\ cm^2)$: 149; Mass Spectrum (FAB) for $(C_{24}H_{32}N_4O_4Fe)^+$ m/z = 496.2; Anal. calcd for $C_{24}H_{32}N_4O_4FeI$: C, 46.25; H, 5.17; N, 8.99 found: C, 46.13; H, 5.22; N, 8.97.

((4,6-di-OMe-R-ENBPI)Fe)$^+$I$^-$ (4b)

Using Method B involving H_3DMabi ligand **4** (110 mg, 0.17 mmol) and Fe(acac)^3 (60 mg, 0.17 mmol) and KI (28 mg) yielded **4b** (90 mg, 0.13 mmol, 77 %). IR (KBr, cm^{-1}): 3420 υ(O-H), 3110 υ(N-H), 3000-2850 υ(C-H), 1610 υ(C=N), 1550 δ(N-H), 1595, 1460, 1430 υ(C=C); $\kappa(\Omega^{-1}\ mol^{-1}\ cm^2)$: 127; Mass Spectrum (FAB) for $(C_{26}H_{36}N_4O_6Fe)^+$ m/z = 556.2; Anal. calcd for $C_{26}H_{36}N_4O_6FeI \cdot 0.5\ H_2O$: C, 45.11; H, 5.39; N, 8.09 found: C, 45.54; H, 5.84; N, 7.64.

((3-OMe-R-ENBPI)Ga)$^+$I$^-$ (3c)

Using Method B involving H_3Mabi ligand **3** (140 mg, 0.24 mmol), Ga(acac)^3 (89 mg, 0.24 mmol) and KI (40.3 mg) yielded **3c** (125mg, 0.19 mmol, 80%). ^{1}H NMR (300 MHz, DMSO-d^6) δ: 1.62 (m, 2H), 1.95 (m, 2H), 2.72 (m, 2H), 3.02 (m, 4H), 3.10-3.65 (m, 6H), 3.81 (s, 6H, OCH_3), 5.18 (bs, 2H, NH), 6.62 (t, 2H, Ar-H), 6.85 (d, 2H, Ar-H), 7.05 (d, 2H, Ar-H), 8.28 (s, 2H, CH=N); IR (KBr, cm^{-1}): 1635 υ(C=N); $\kappa(\Omega^{-1}\ mol^{-1}\ cm^2)$: 137; Mass Spectrum (FAB) for $(C_{24}H_{32}N_4O_4Ga)^+$ m/z = 509.3; Anal. calcd for $C_{24}H_{32}N_4O_4GaI \cdot 2H_2O$: C, 42.82; H, 5.39; N, 8.32 found: C, 42.85; H, 5.31; N, 8.35.

((4,6-di-OMe-R-ENBPI)Ga)$^+$I$^-$ (4c)

Using Method B involving H_3DMabi ligand **4** (130 mg, 0.19 mmol) and Ga(acac)^3 (72 mg, 0.19 mmol) and KI (32 mg) yielded **4c** (110 mg, 0.16 mmol, 81%). ^{1}H NMR (300 MHz, DMSO-d^6) δ: 1.55 (m, 2H), 1.94 (m, 2H), 2.65 (m, 2H), 2.95 (m, 4H), 3.35 (m, 4H), 3.67 (t, 2H), 3.75 (s, 6H), 3.78 (s, 6H), 4.96 (bs, 2H, NH), 5.87 (2H, Ar-H), 5.96 (2H, Ar-H), 8.28 (s, 2H, CH=N); IR (KBr, cm^{-1}): 1610 υ(C=N); $\kappa(\Omega^{-1}\ mol^{-1}\ cm^2)$: 133; Mass Spectrum (FAB) for $(C_{26}H_{36}N_4O_6Ga)^+$

m/z = 569.1; Anal. calcd for $C_{26}H_{36}N_4O_6GaI \cdot 2H_2O$: C, 42.59; H, 5.49; N, 7.64 found: C, 42.93; H, 5.38; N, 7.78.

((3-OMe-R-ENBPI)In)$^+$$NO_{3-}$ (3d) (Footnote 1)

Using Method A involving H_3Mabi ligand **3** (132 mg, 0.23 mmol), potassium hydroxide (3M, 230µl) and $In(NO_3) \cdot 5H_2O$ (89 mg, 0.23 mmol) on final washings with ether containing 15% of methanol yielded **3d** (100 mg, 0.16 mmol, 71%). ^{1}H NMR (300 MHz, DMSO-d^6) δ: 1.75 (m, 2H), 2.12 (m, 2H), 2.65 (m, 2H), 3. 1 0 (m, 4H), 3.46 (m, 2H), 3.67 (m, 2H), 3.75 (s, 6H, OCH_3), 4.17 (t, 2H), 5.22 (bs, 2H, NH), 6.54 (t, 2H, Ar-H), 6.79 (d, 2H, Ar-H), 6.97 (d, 2H, Ar-H), 8.34 (s, 2H, CH=N); IR (KBr, cm^{-1}): 1626 υ(C=N), 1280-1244 b υ (NO_{3-}); κ(Ω^{-1} mol^{-1} cm^2): 110; Mass Spectrum (FAB) for $(C_{24}H_{32}N_4O_4In)^+$ m/z = 555.0; Anal. calcd for $C_{24}H_{32}N_4O_4InI \cdot KCI$ (Footnote 1): C, 38.09; H, 4.26; N, 7.40 found: C, 38.39; H, 4.86; N, 7.08.

((4,6-di-OMe-R-ENBPI)In)$^+$$I^-$ (4d)

Using Method B involving H_3DMabi ligand **4** (118 mg, 0.17 mmol), In(acac)^3 (73 mg, 0.17 mmol) and KI(30 mg) resulted in **4d** (100 mg, 0.13 mmol, 76%). ^{1}H NMR (300 MHz,DMSO-d^6) δ: 1.70 (m, 2H), 2.10 (m, 2H), 2.55 (m, 2H), 3.05 (m, 4H), 3.40 (m, 4H), 3.72 (s, 6H, OCH_3), 3.76 (s, 6H, OCH_3), 3.94 (t, 2H), 5.14 (bs, 2H, NH), 5.83 (2H, Ar-H), 5.94 (2H, Ar-H), 8.34 (s, 2H, CH=N); IR (KBr, cm^{-1}): 1608 υ(C=N), κ(Ω^1 mol^1 cm^2): 174 ; Mass Spectrum (FAB) for $(C_{26}H_{36}N_4O_6In)^+$ m/z= 615.1; Anal. calcd for $C_{26}H_{36}N_4O_6InI \cdot 2H_2O$: C, 40.12; H, 5.18; N, 7.20 found: C, 39.82; H, 5.01; N, 7.47.

Cell Culture and Cytotoxicity Assays

Monolayers of parental KB-3-1 and multidrug-resistant KB-8-5 cell lines were routinely grown in DMEM (GIBCO, Grand Island, NY) supplemented with L-glutamine (2 mM), penicillin/streptomycin (0.1%) and heat-inactivated fetal

bovine serum (10%) in the absence and presence of 10 ng/ml colchicine (Sigma Chemical Co.), respectively. Multidrug-resistant cells were cultured in drug-free media for 96 h prior to cytotoxicity assays. Cytotoxicity potencies of R-ENBPI metal-complexes, metal salts or colchicine were determined in 96-well microtiter plates as described (Piwnica-Worms, D. *et al., Cancer Res. 53*:1-8 (1993)). Cells (5,000/well) were plated in media containing fetal bovine serum (5%) and allowed to recover for 4 h. The indicated concentrations of R-ENBPI complex, metal(III) nitrate with matched vehicle or a cytotoxic concentration of colchicine (10 μg/ml; 25 μM) were added in triplicate wells for each cell line. Cells were then incubated for 72 h under normal growth conditions (37°C, 5% CO_2 atmosphere).

Cell survival was assayed using sulforhodamine B (SRB) as previously described with slight modification (Skehan, P. *et al., J. Natl. Cancer Inst. 82*:1107-1112 (1990)). Briefly, cells were fixed in 10% trichloroacetic acid for 60 min at 4°C, washed 5 times with tap water, and allowed to air-dry overnight (25°C). Protein precipitates were then stained with 0.4% SRB in 1% acetic acid for 20 min at room temperature. Excess SRB was removed with four 1% acetic acid washes, plates were air-dried (25°C), and then the stain was redissolved in IO mM unbuffered TFis base.

Quantitation was performed on an ELISA plate reader using an absorption difference technique (490nm - 450 nm). Survival was expressed as the percentage of surviving cells relative to growth in media containing drug vehicle alone (ligand **3**, 1 % ethanol; ligand **4** and metal(III) nitrates, 0.85% ethanol/0.15% DMSO). LC_{50} determinations were obtained from the cell survival curves by computer fitting with a sigmoid equation:

$$S = \{(S_{max} - S_{min})/(1 + (C/LC50)\gamma)\} + S_{min},$$

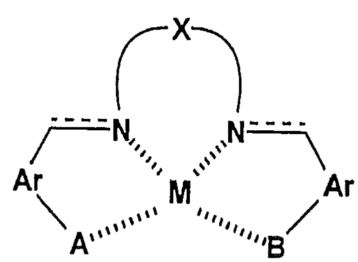
where S is cell protein, S_{max} is cell protein in control buffer, S_{min} is residual cell protein at highest drug toxicity, γ is the slope, C is cytotoxic agent concentration

and LC_{50} represents the half-maximal cytotoxic concentration. Paired data were compared by the Student's *t* test (Glantz, S.A., *Primer of Biostatistics,* 2nd ed., McGraw-Hill, Inc., New York, p. 379 (1987)). Most analogs were tested in at least two separate culture experiments with essentially identical results.

From the foregoing description, one skilled in the art can easily ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions without undue experimentation.

What is Claimed is:

1. A multidentate cationic metal complex having the following formula:



I

wherein

M is Fe, In, Ga or Al;

the dashed lines represent independently a single or a double bond;

the hatched lines represent coordination to the metal cation (M);

A and B are oxygen, or in the case where Ar is 2-pyridyl or 2-pyrrolyl, a shared pair of electrons on the nitrogen;

Ar is optionally substituted phenyl, optionally substituted naphthyl, optionally substituted 2-pyridyl or optionally substituted 2-pyrrolyl; and

X is $(CHR^2)_p[NR^3(CHR^4)_q]_r$, wherein p and q are independently 1, 2, 3, 4, 5 or 6; r is 0, 1 or 2; and R^2, R^3 and R^4 are independently hydrogen, lower alkyl or phenyl, or two adjacent R^2 or R^4 groups represent a double bond or a fused benzene ring where p or q, respectively, is greater than 2; with the proviso that where r is 1 or 2, then there are 1 or two additional sites of coordination to the metal,

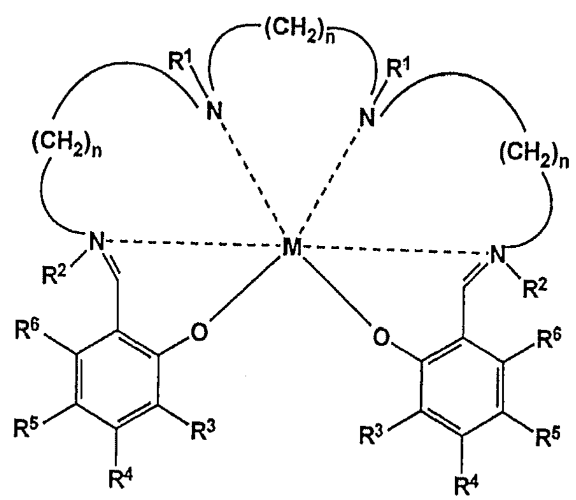
optionally with: proviso (A), wherein at least one of the substituents of Ar comprises boron, or with proviso (B), wherein at least one of substituents of Ar comprises a linkage to a pharmaceutically active substituent.

2. A multidentate cationic metal complex according to claim 1, wherein X is alternatively an optionally substituted phenyl.

3. A multidentate cationic metal complex according to claim 1, wherein the complex has overall positive charge.

4. A multidentate cationic metal complex according to claim 3, wherein the complex further contains -A⁻, wherein -A⁻ is a pharmaceutically acceptable anion.

5. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *V*:



V

wherein:

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

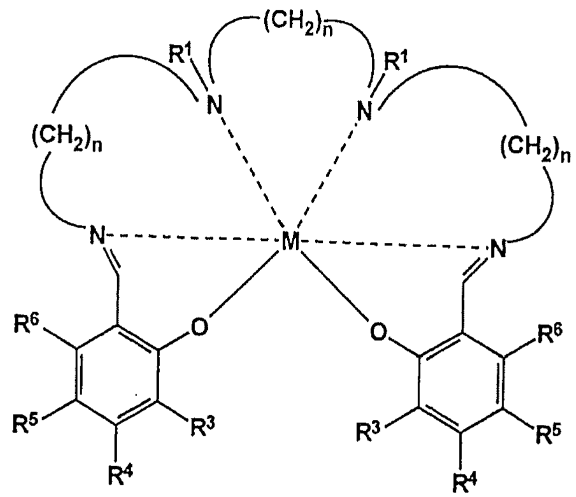
the dashed lines represent coordination to the metal cation (M);

R^1 and R^2 are independently hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group; and

n = 2, 3, 4, 5 or 6.

6. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *VI*:



VI

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

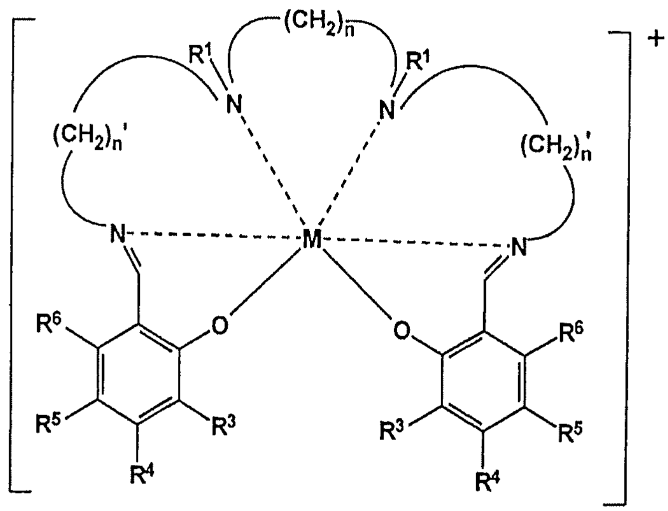
the dashed lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group; and

n = 2, 3, 4, 5 or 6.

7. A multidentate cationic metal complex according to claim 6, wherein said multidentate cationic metal complex is according to formula *VII*:



VII

wherein:

M is Fe^{+3} or Al^{+3};

the dashed lines represent coordination to the metal cation (M);

R^3 is OCH_3 or OC_2H_5;

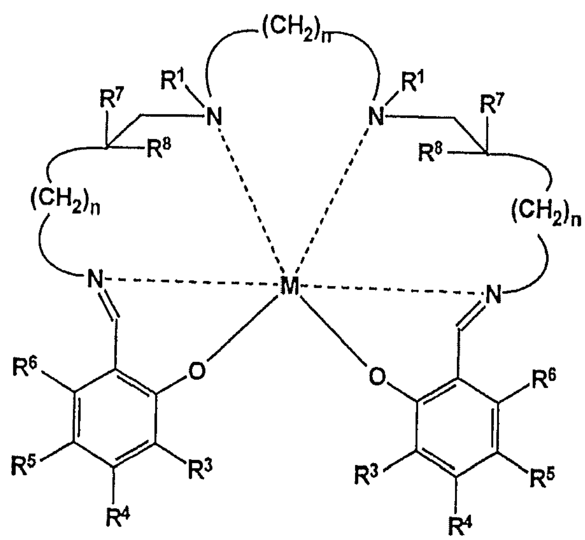
R^4 and R^6 are OCH_3;

R^1 and R^5 are H;

n is 2; and

n′ is 3.

8. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *VIII*:



VIII

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent coordination to the metal cation (M);

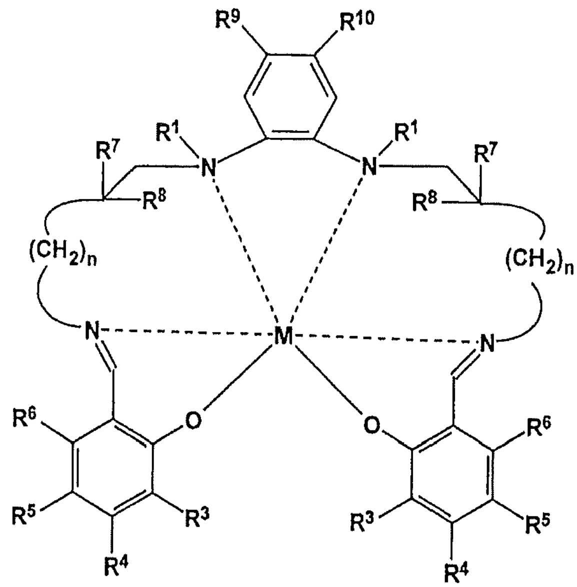
R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7 and R^8 are independently hydrogen or alkyl; and

n = 1, 2, 3, 4, 5 or 6.

9. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *IX*:



IX

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent coordination to the metal cation (M);

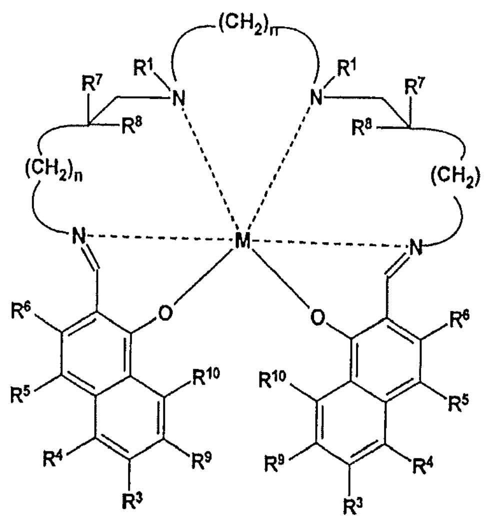
R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7, R^8, R^9 and R^{10} are independently hydrogen or lower alkyl; and

n = 1, 2, 3, 4, 5 or 6.

10. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *X*:



X

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

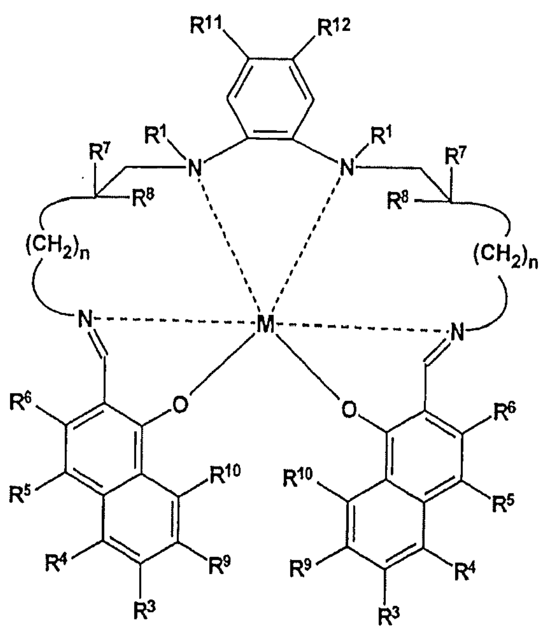
R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group;

R^7 and R^8 are independently hydrogen or alkyl;

R^9 and R^{10} are independently hydrogen, a lower alkyl, a lower alkoxy, halo or a nitro group; and

n = 1, 2, 3, 4, 5 or 6.

11. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *XI*:



XI

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent coordination to the metal cation (M);

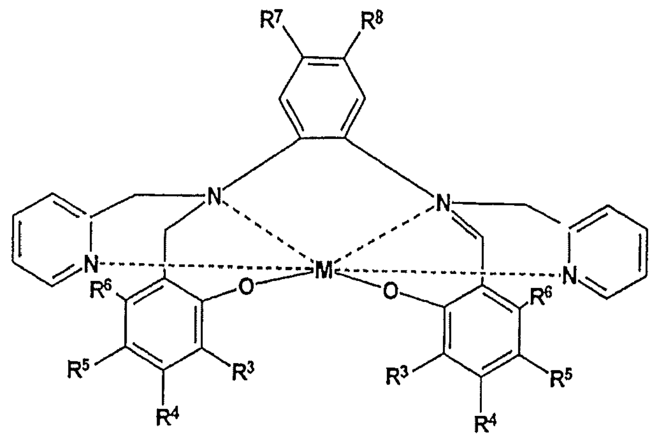
R^1 is hydrogen or alkyl;

R^3, R^4, $R^{5,}$ R^6, R^9 and R^{10} are independently a lower alkoxy, halo or nitro group;

R^7, R^8, R^{11} and R^{12} are independently hydrogen or lower alkyl; and

n = 1, 2, 3, 4, 5 or 6.

12. A multidentate cationic metal complex according to claim 1, wherein said multidentate cationic metal complex is according to formula *XII*:



XII

wherein

M is Fe^{+3}, In^{+3}, Ga^{+3}, or Al^{+3};

the dashed lines represent coordination to the metal cation (M);

R^1 is hydrogen or alkyl;

R^3, R^4, R^5 and R^6 are independently a lower alkoxy, halo or nitro group

R^7 and R^8 are independently hydrogen or alkyl; and

n = 1, 2, 3, 4, 5 or 6.

13. A pharmaceutical composition comprising the complex of claim 1 and a pharmaceutically acceptable carrier.

14. A method of treating at least one disease selected from the malaria, cancer, diseases attributable to the multidrug family of transporters, comprising administering to a patient in need of such treatment an effective amount of a multidentate cationic metal complex according to claim 1.

15. The method of claim 14, wherein said disease is malaria and said multidentate cationic metal complex is coadministered substantially simultaneously with at least one active antimalarial substance.

16. A method of potentiating photodynamic therapy in the treatment of cancer cells, comprising administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to claim 1.

17. A method of treating of cancer by boron-neutron therapy, comprising administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to claim 1, with proviso (A), wherein at least one of the substituents of Ar comprises boron.

18. A method of enhancing the oral absorption or cell accumulation of a pharmacologically active substances, comprising administering to an animal in need thereof an effective amount of a multidentate cationic metal complex according to claim 1.

19. The method of claim 18, wherein said pharmacologically active substance is selected from the group consisting of a peptide, oligonucleotide, peptidomimetic, nucleic acid analog and a derivative thereof.

20. The method of any of claims 14-19, wherein said compound is administered to said animal at an effective dosage of from about 50 mg to 500 mg per day.

P. falciparum GROWTH INHIBITION:
CONCENTRATION-EFFECT CURVES OF SUBSTITUTED HEXADENTATE MENPBI-FE(III) COMPLEXES

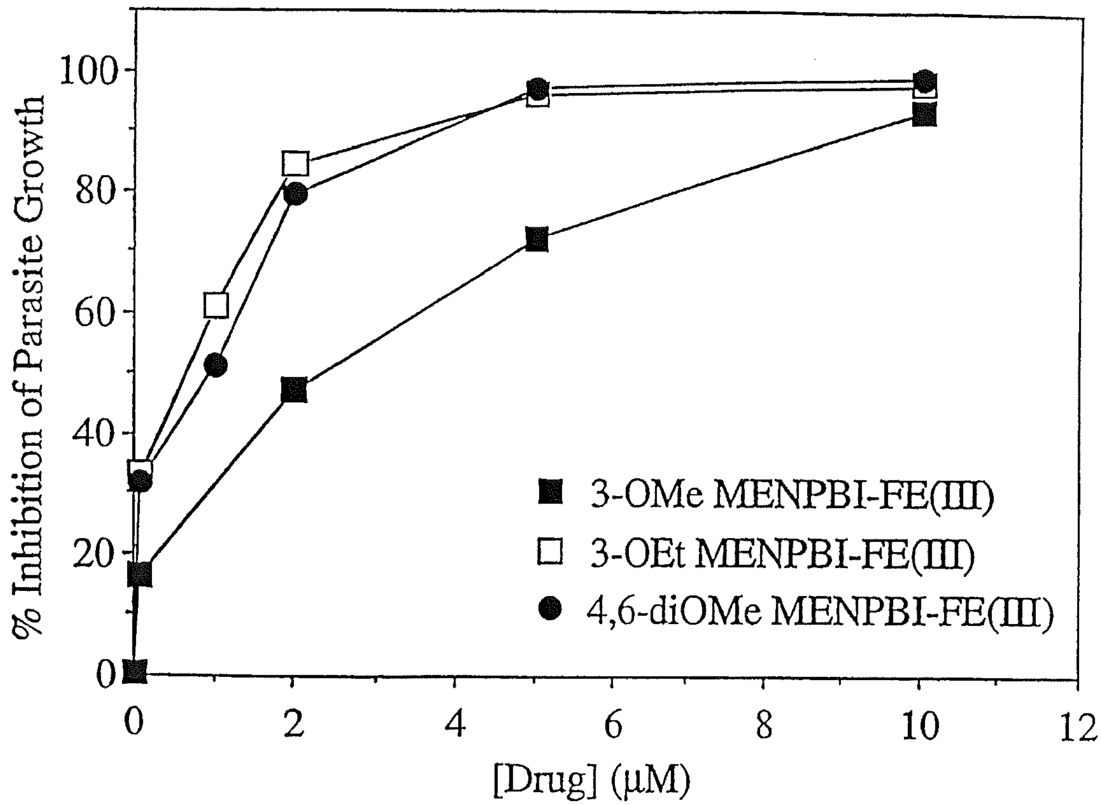


Figure 1

Cytotoxicity of a MENPBI Complex in KB-3-1 and KB-8-5 Cells

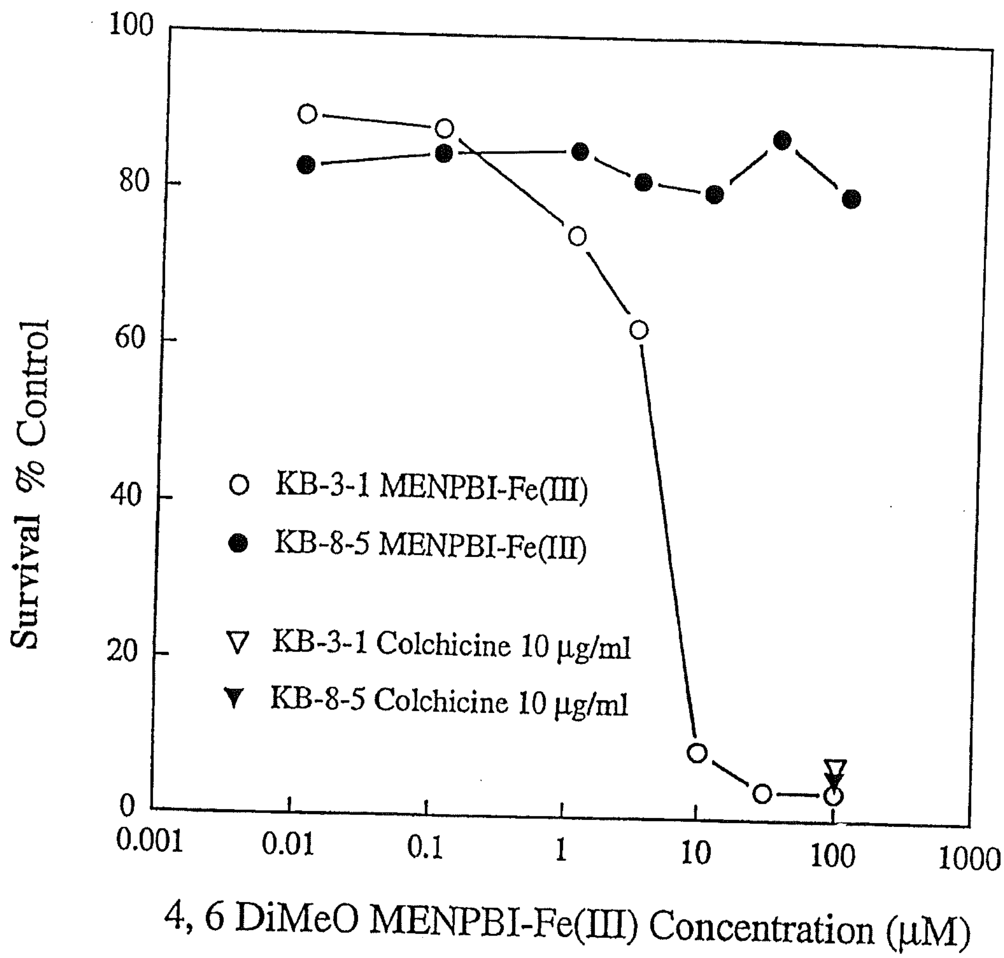
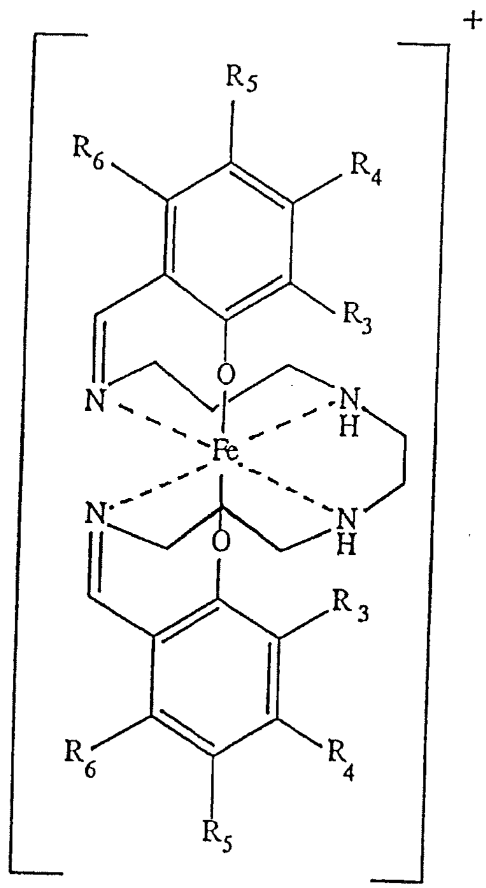


Figure 2



2

$R^3 = OCH_3$; R^4, R^5, $R^6 = H$

R^4, $R^6 = OCH_3$; R^3, $R^5 = H$

Figure 3

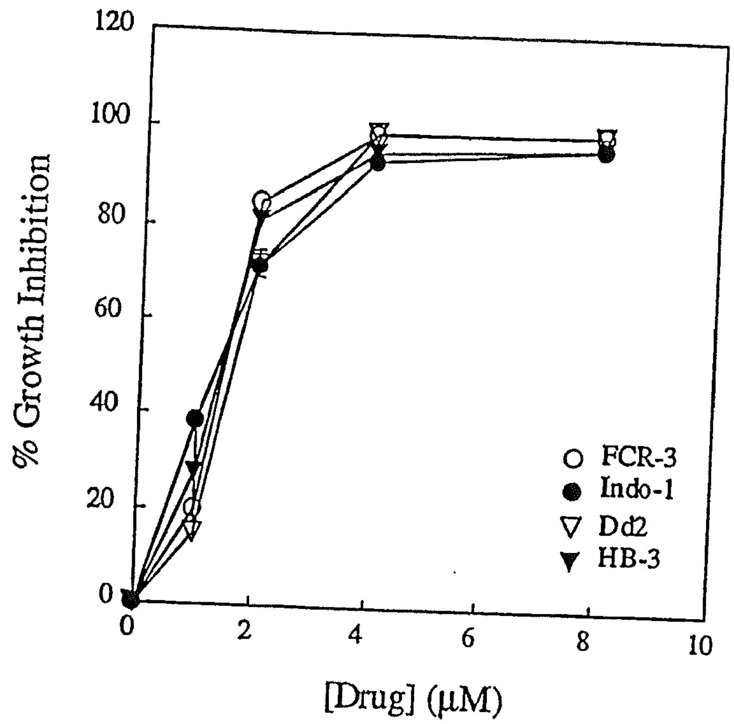

120
100
80
60
40
20
0
% Growth Inhibition
0
2
4
6
8
10
[Drug] (μM)
FCR-3
Indo-1
Dd2
HB-3

Figure 4A

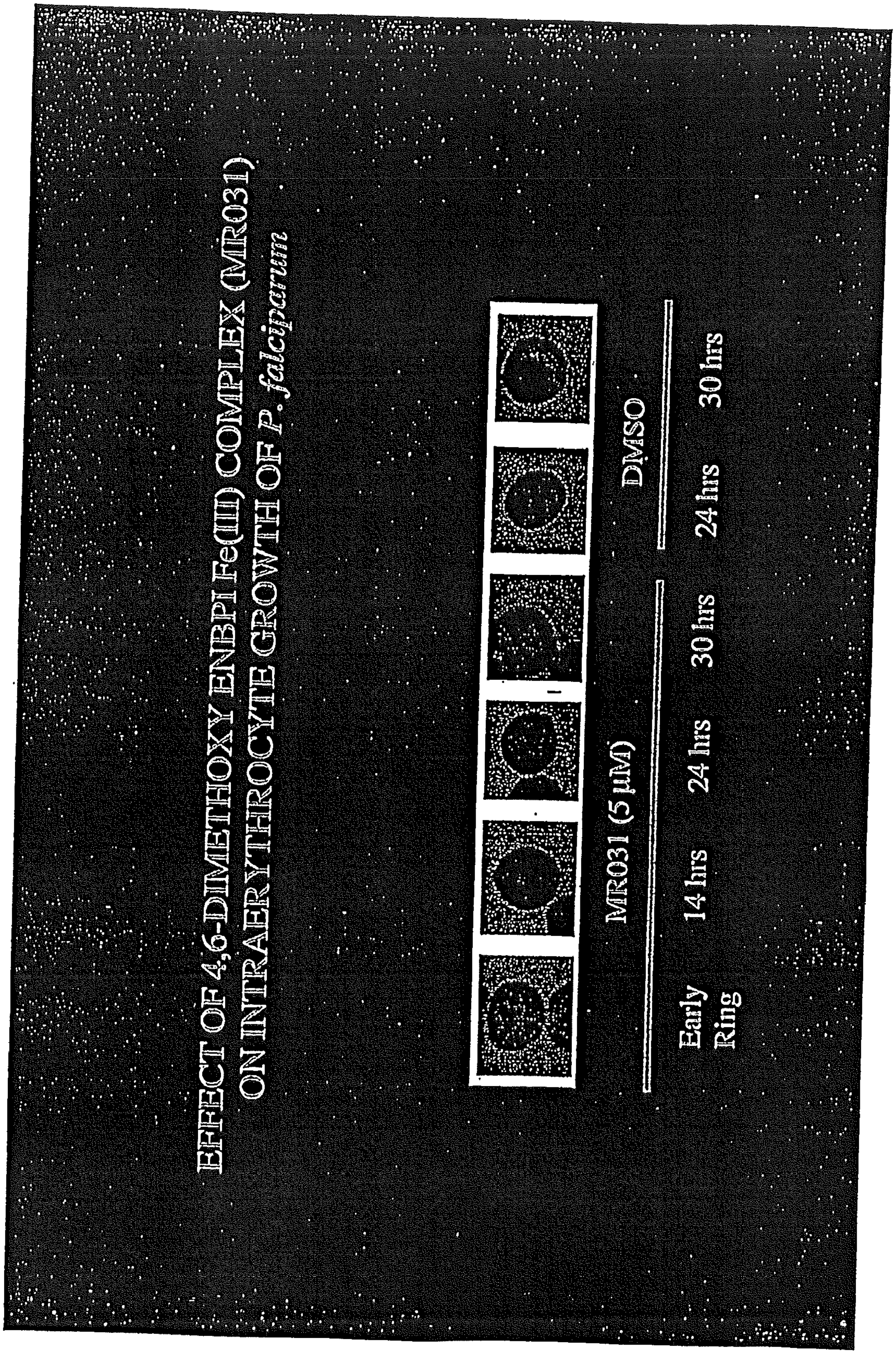

EFFECT OF 4,6-DIMETHOXY ENBPI Fe(III) COMPLEX (MR031)
ON INTRAERYTHROCYTE GROWTH OF P. falciparum
MR031 (5 μM)
DMSO
Early Ring
14 hrs
24 hrs
30 hrs
24 hrs
30 hrs

Figure 4B

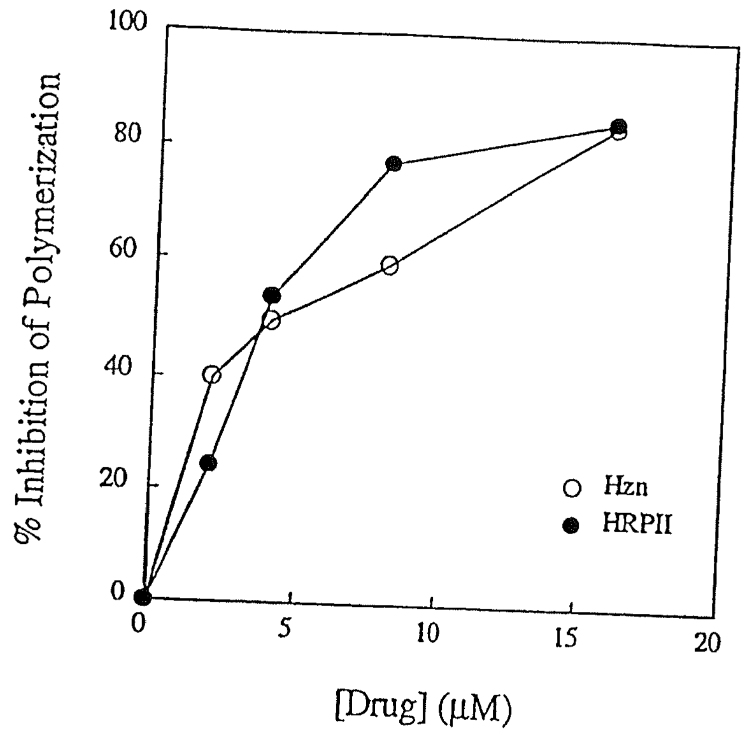
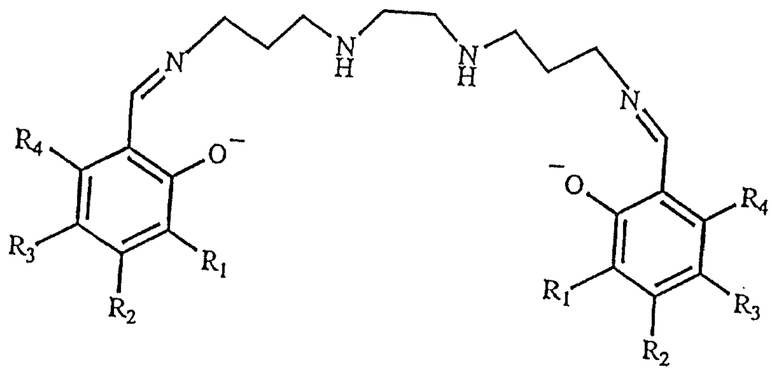

100
80
60
40
20
0
% Inhibition of Polymerization
0
5
10
15
20
[Drug] (μM)
Hzn
HRPII

Figure 5



1

$R^3 = OCH_3$; R^4, R^5, R^6 = H
R^4, $R^6 = OCH_3$; R^3, R^5 = H

Figure 6

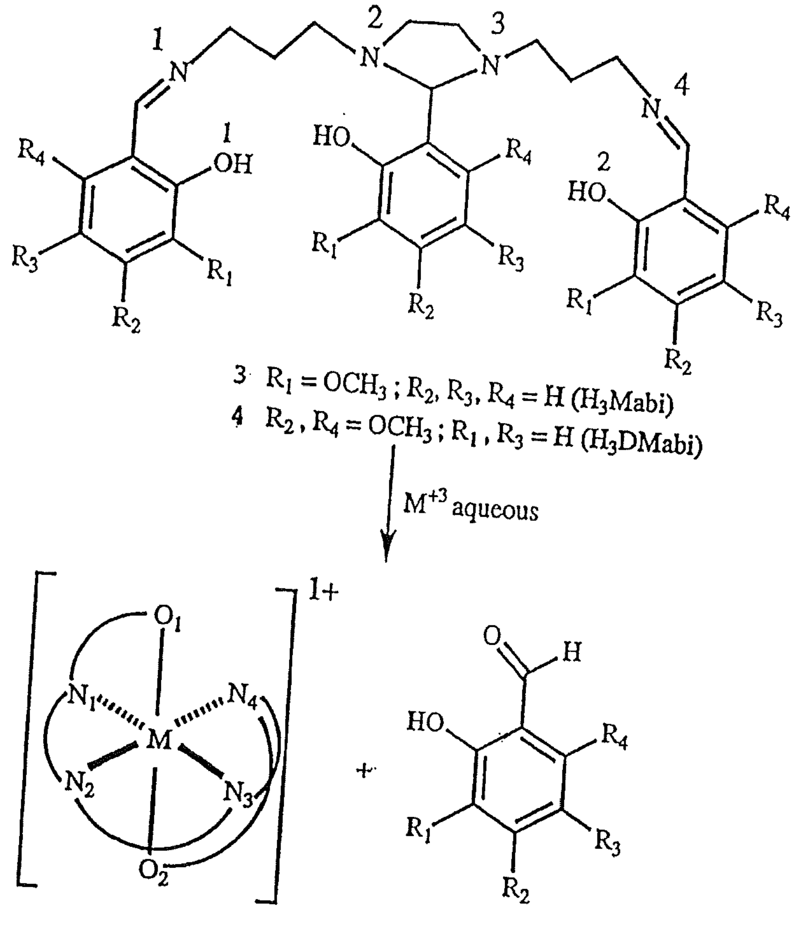

3 $R_1 = OCH_3$; $R_2, R_3, R_4 = H$ (H_3Mabi)
4 $R_2, R_4 = OCH_3$; $R_1, R_3 = H$ (H_3DMabi)
M^{+3} aqueous
1+

Figure 7

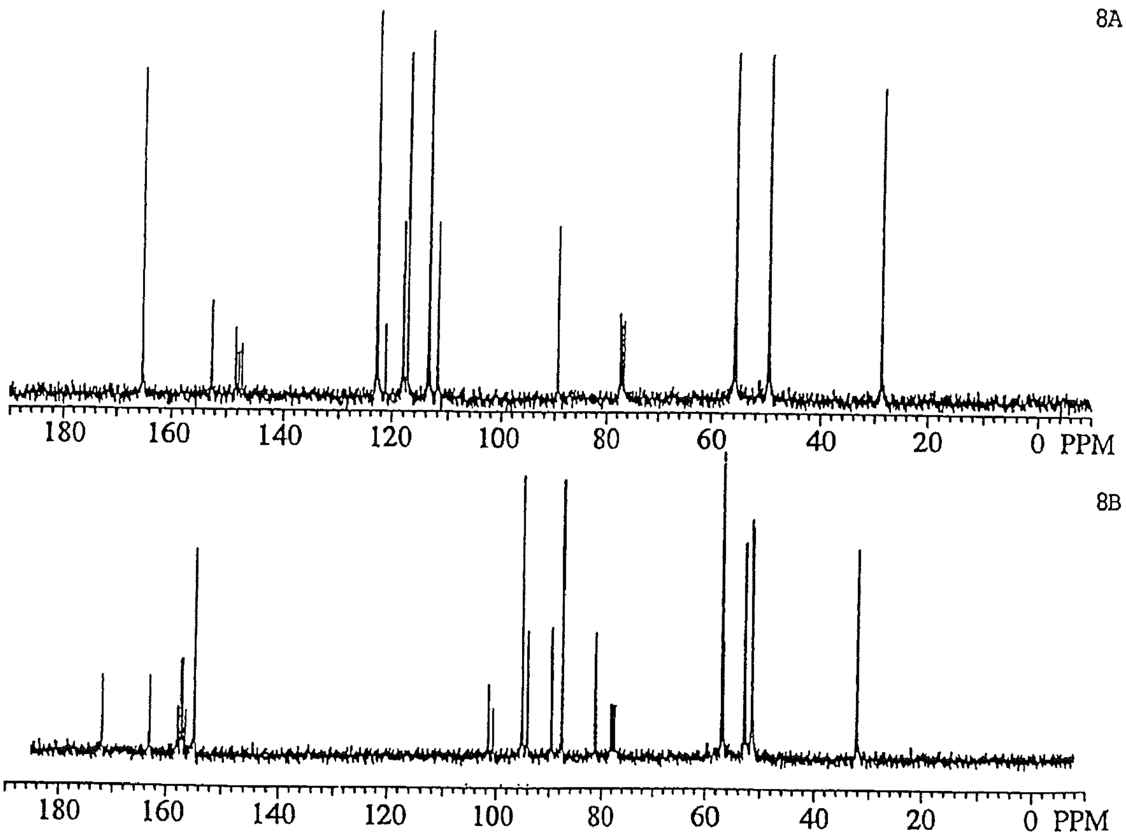

8A
180
160
140
120
100
80
60
40
20
0 PPM
8B
180
160
140
120
100
80
60
40
20
0 PPM

Figure 8A-B

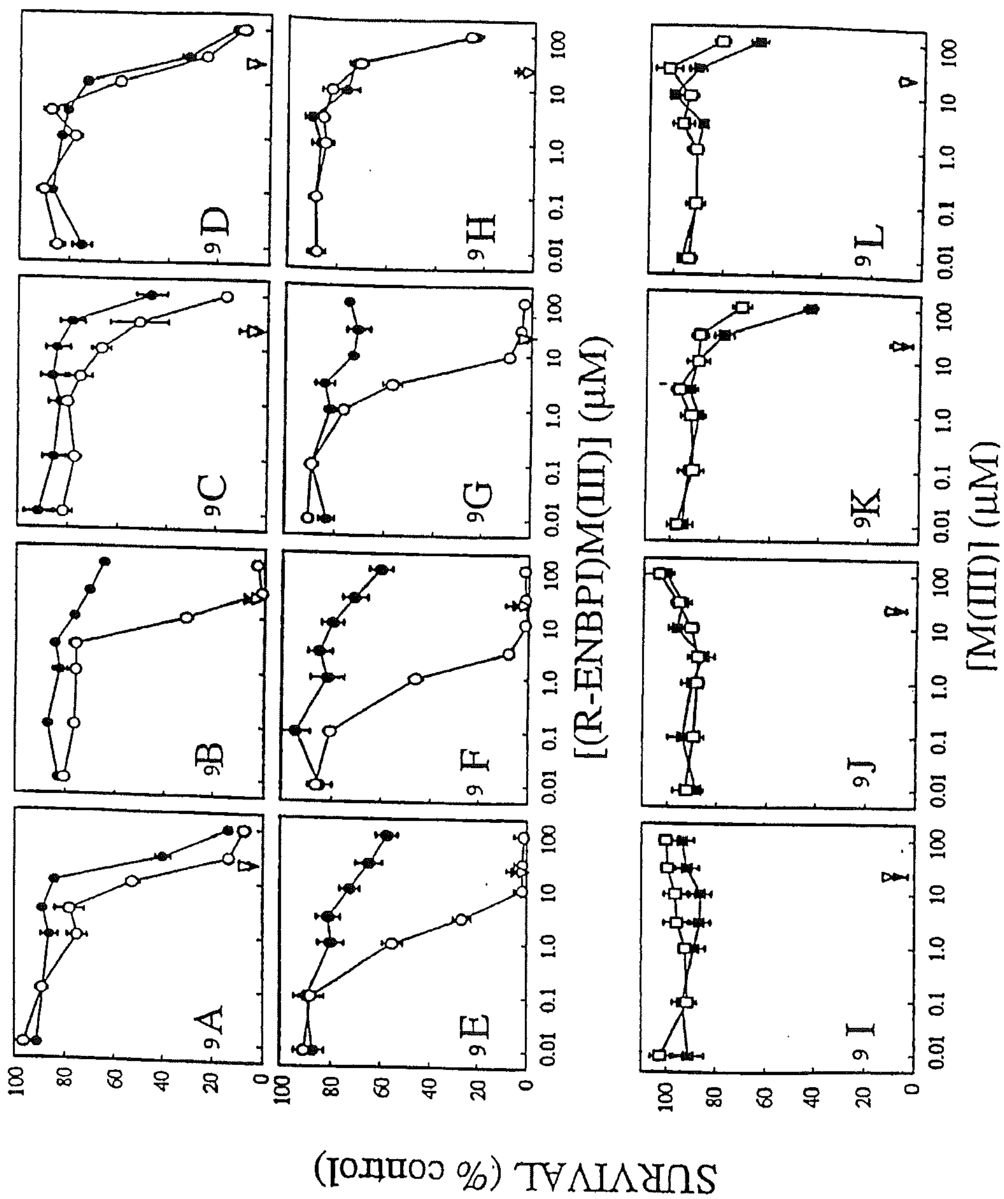

SURVIVAL (% control)
[(R-ENBPI)M(III)] (μM)
[M(III)] (μM)
9A
9B
9C
9D
9E
9F
9G
9H
9I
9J
9K
9L

Figure 9A-L

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/10079

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/33, 31/555; C07F 5/00, 5/06, 7/10, 15/02
US CL :514/184, 188, 191; 546/2, 6; 548/402, 403; 556/1, 32

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/184, 188, 191; 546/2, 6; 548/402, 403; 556/1, 32

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,281,578 A (F.C. BRADLEY ET AL.) 25 January 1994, see column 1, lines 46-70 and column 2, lines 1-70.	1-6 and 9-13
X	ADDISON et al. Some Iron(III) Complexes with Polydentate Schiff Base Ligands. Inorganica Chimica Acta. 1988, Vol. 147, pages 61-64, especially pages 61 and 62.	1-12
X	EVANS et al. Complexes Of Water-Soluble Hexadentate Schiff Base Ligands With A Number Of Metal Ions. Polyhedron. 1988, Vol. 7, pages 1881-1889, especially pages 1882-1883.	1-12

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 AUGUST 1996	03 OCT 1996

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	PAUL F. SHAVER
Facsimile No. (703) 305-3230	Telephone No. (703) 308-1235

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TWEEDLE et al. Variable Spin Iron (III) Chelates with Hexadentate Ligands Derived from Triethylenetetramine and Various Salicylaldehydes. Synthesis, Characterization, and Solution State Studies of a New $T^2 \rightleftarrows {}^6$ A Spin Equilibrium System. Journal of the American Chemical Society. 04 August 1976, Vol. 98, No. 16, pages 4824-4834, especially pages 4824-4827.	1-12
X	WONG et al. Hexadentate N_4O_2 Amine Phenol Complexes of Gallium and Indium. Inorganic Chemistry. 1995, Vol. 34, No. 1, pages 93-101, especially pages 94-96.	1-12